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KINETIC MODELING AND ITS APPLICATION IN THE BIOPHARMACEUTICAL INDUSTRY

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KINETIC MODELING AND ITS APPLICATION IN THE BIOPHARMACEUTICAL INDUSTRY

by

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Declarations

Declaration of originality

As the author of this thesis, I (Peifeng Tang) declare that this work herein presented is my own work, original and documented in my own words. All relevant contributions have been acknowledged and referenced in the text.

Declaration of copyright

Acknowledgements

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ABBREVIATIONS

ADC, Antibody drug conjugate

ADCC, antibody-dependent cell-mediated cytotoxicity

BLA, Biologic license application

CAR-T, chimeric antigen receptor T-cell immunotherapy

CFDA, China Food and Drug Administration

CHO, Chinese Hamster Ovary

CHO-A, Chinese Hamster Ovary cell line A

CHO-B, Chinese Hamster Ovary cell line B

CMC, Chemistry, Manufacturing and Control

CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats

dhfr, dihydrofolate reductase

DoE, Design of Experiment

ELISA, Enzyme-linked immunosorbent assay

EMA, European Medicines Agency

FDA, US Food and Drug Administration

FMAT, Fluorometric microvolume assay

FACS, Fluorescence-activated cell sorting

GMP, Good Manufacturing Practice

GS, glutamine synthetase

HMW, High molecular weight - 12 -

ICH, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use

IND, investigational new drug

KNF, Koshland-Nemethy-Filmer

mAb, Monoclonal antibody

M-M, Michaelis-Menten

MWC, Monod-Wyman-Changeux

NAD+, nicotinamide adenine dinucleotide

NADH, nicotinamide adenine dinucleotide hydrogen

QbD, Quality by Design

PIC/S, Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme

TS, Temperature shift

VCD, Viable cell density

Abstract

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It has been well recognized that biologics are efficient for cancer and immune disease treatment. Kinetic modeling is to mathematically model or to quantitatively illustrate how reactions occur in a biological or chemical process. A systematic study and understanding of kinetic model and its application in the biopharmaceutical industry are important for both scientific research and industrial technology development.

This work consists of six chapters. First, a review of kinetic modeling and its application for cell culture was introduced. Second, the current status of biologics development and screening strategies of biomarkers and indications were discussed. Third, one experimental and kinetic modeling study for the temperature effects and temperature shift strategy development was presented. Forth, novel kinetic models were built up and applied to elucidate lactate dehydrogenase catalyzed reactions, which is a crucial metabolic process within tumor cells and Chinese hamster ovary cells. In the fifth chapter, strategies of biologics quality control via process development were briefly summarized. And finally, summary and outlook were made based on the above five chapters.

Though kinetic modeling is not a FDA request tool, kinetic data are required for regulation approval of new drug discovery and process development. These data are applicable for a rapidly screening of the best biologics and the optimized manufacturing process with little extra cost via kinetic modeling. This work is potentially beneficial for speeding up and better understanding of the current biologics development in biopharmaceutical industry.

Key Words: Chinese Hamster Ovary (CHO) cell, cell culture, kinetic modeling, biomarker screening, cancer treatment, lactate dehydrogenase, temperature shift, quality control

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Introduction

Biologics is applicable in treating cancer and immune system diseases. From the first mAb OKT3 approved by FDA in 1986 to the recent approved chimeric antigen receptor T-cell immunotherapy (CAR-T) on August 30th 2017, the biopharmaceutical industry has been expanding at an ever faster pace in the recent three decades. To get approved for commercial use, complex pre-clinical development and clinical studies are needed to ensure the safety, stability, and efficacy of the biologics, which typically lasts 10 to 12 years. Thus, efficient methods to illustrate the biologics production process, enzyme and cell properties, are helpful for biologics discovery and development, as well as for potentially accelerating the commercialization process.

Biologics screening and process developments are two major tasks in the pre-clinical stage. Current strategies are mainly based on two sets of criteria:

A) Static parameters evaluation. For example, peak VCD, final titer, final quality profile are frequently used for process development.

B) Thermodynamic parameter evaluation. For example, binding affinity is typically used for antibody-dependent cell-mediated cytotoxicity evaluation to screening the best performance biologics.

However, little process information can be directly reflected from the above two strategies, without which the process development and biologics screening would be heavily experience dependent as well as risky in missing the best options. Herein additional kinetic modeling is serving as a tool to make up these drawbacks. Kinetic

modeling is dependent on the understanding of the enzymatic mechanism within cells, which can promote new drug discovery, guide clinical study, efficacy explanation, and enhance cell metabolism understanding.

In this dissertation, an attempt on the systematic study of kinetic modeling and its application to biologic screening, process development and enzymatic mechanism understanding were made. It aimed at benefiting the biopharmaceutical industry to accelerate the commercialization process, as well as to reduce the cost and labor.

Chapter 1

Kinetic Modeling of CHO Cell Culture: principle and Application

1.1. Abstract

As a host for therapeutic proteins, Chinese Hamster Ovary cell is widely utilized in the mainstream biopharmaceutical industry. Cell culture process development plays an important role stepping from lab research to manufacturing. Among different mathematic tools, kinetic modeling is commonly achieved through analyzing cell culture data to design process parameters, optimize medium, and scale up the reactors. In this review, we examined key factors for upstream process development, and summarized currently used kinetic modeling strategies. Meanwhile, one original example of kinetic modeling application in optimizing cell culture performance is presented. This review aims at providing a comprehensive understanding of kinetic modeling and its application for cell culture process development.

Keywords: Chinese hamster ovary (CHO), kinetic modeling, cell culture, therapeutic protein

Abbreviations: **CHO**, Chinese hamster ovary; ***dhfr***, dihydrofolate reductase; **FDA**, Food and Drug Administration; **GS**, glutamine synthetase; **mAb**, monoclonal antibody; **M-M**, Michaelis-Menten; **MSX**, methione sulphoximine; **MWC**, Monod-Wyman-

Changeux; **VCD**, viable cell density;

1.2. Introduction

Chinese Hamster Ovary (CHO) cells, as one type of mammalian cells, were first introduced in 1957 by Theodore T. Puck when fibroblast cells were recovered from the cultured ovarian cells of a partially inbred Chinese hamster [1]. CHO cells have since become the preferred host for therapeutic protein production. Since the first biologics gained approval in 1986 [2], Around 75 monoclonal antibodies (mAbs) and fusion protein products have been approved in the US or Europe with about 70 % of them produced by CHO cells as of July 2017 [3-6]. The total sales of these top 10 biologics valued at $ 61.2 billion [5], while 500 antibody-based biologics were in the clinical stage in 2016 [6].

As one bridge connecting lab research to manufacturing, cell culture process development is an important step to biologics commercialization [7]. Significant progresses in CHO cell line engineering, media development, and process control, have enabled cell culture to achieve the titers of 3-6 g/L, which is now common for typical industrial fed-batch cell culture [8-10]. However, many challenges still remain in this field. First, higher titer will likely become necessary given the limited manufacturing capacity and high demands for multiple products. Second, the proteins produced need to have consistently specific product quality attributes for safety and efficacy. Finally, cell culture processes need to be robust and scalable from the lab to manufacturing scales [11]. The current paradigm in cell culture needs to evolve to address these

challenges.

While the current practice of cell culture process development is heavily empirical and may result to inefficiency in some cases, kinetic modeling performs as a mathematic tool to efficiently evaluate, analysis and optimize cell culture processes.[12] With structured or unstructured kinetic models, cell culture performance can be quantitatively evaluated and predicted. Meanwhile, it allows multiple process variations to be optimized simultaneously. This leads to fast critical process parameter evaluation and accelerates process development speed, which potentially benefits the automation system set-up in cell culture, reduces the lab work load and saves time and resources [13].

In this chapter, we summarized critical cell culture factors, and reviewed current cell culture kinetic modeling strategies. One kinetic modeling case study on medium optimization was shown, followed by a discussion of the current challenges and perspectives. This work aims at providing a comprehensive understanding of the cell culture kinetic modeling principle and application.

1.3. Overview of CHO cell culture upstream factors

The performance of CHO cell culture are determined by multiple factors working in synergy: cell line, cell culture media, process parameters and bioreactor modes, etc. are the critical ones that impact on the titer and protein quality. Each of them will be discussed in this section.

1.3.1. Cell lines

The first step of recombinant protein expression in mammalian cells is the

transfection of the product transgene to the host cells. Subsequently, hundreds to thousands of individual clones are screened to obtain a few clones with the desired characteristics such as good growth rate, high protein titer and qualified quality attributes. In order to significantly reduce the number of undesired clones, stringent selection pressure is necessary. Fig. 1-1 presented the commercial CHO cell line pedigree since the original cell line in 1957 [14-19]. It indicates that modern cell line development employs either dihydrofolate reductase (*dhfr*) or glutamine synthetase (GS) gene as a basis for clone selection.

Different clones coming out from the same clone screening process can have various performance, even when they are cultured using the same process. Some of the phenotypic differences have been shown to be due to the intrinsic gene expression of the cells [20]. Better understanding of gene-trait relationship will be warranted for more efficient clone screening and process development.

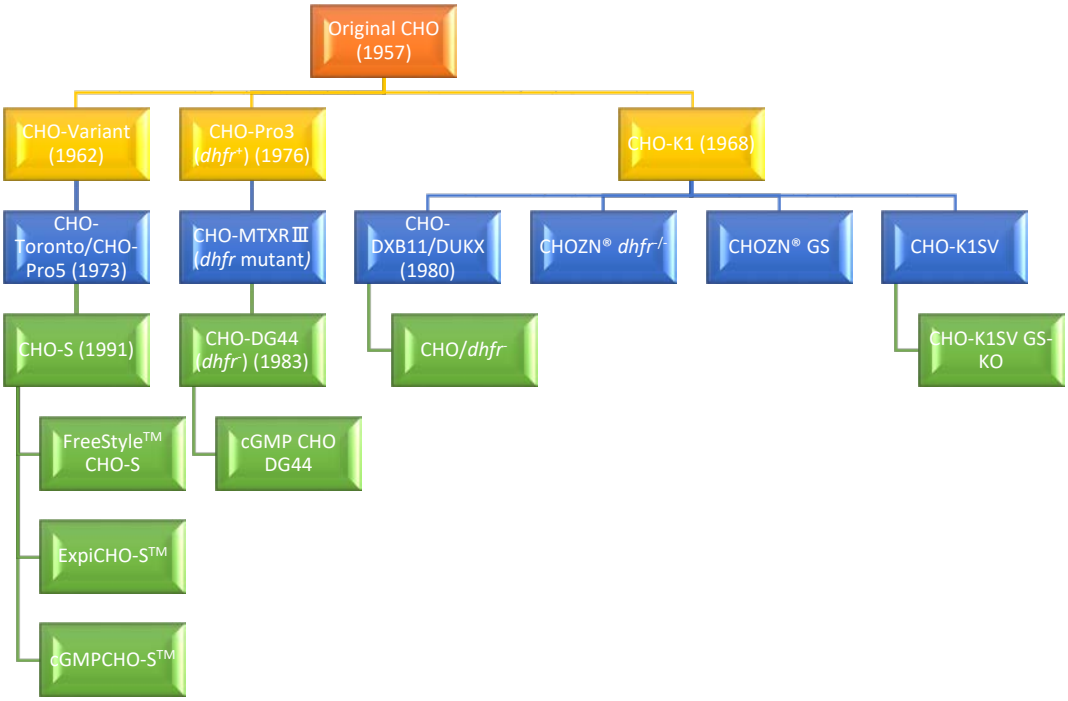


Fig. 1-1. Commercial CHO cell line pedigree.

1.3.2. Medium

An optimized medium provides balanced nutrients to sustain the cell growth and high protein titer expression, while simultaneously reduces accumulation of toxic metabolic byproducts [21, 22]. The first generation of media used in the industry contained serum and/or other undefined components such as hydrolysates to boost cell growth and productivity. However, serum containing media posed potential risks for viral contamination. In addition, it was recognized that lot-to-lot variation of those undefined components can result in inconsistent process performance such as cell growth, titer and product quality [23, 24]. The modern cell culture media contain fully chemically defined components for high process consistency and robustness. This type of media allows a mechanistic understanding of each component, pertinent improvement of productivity and control of the protein quality attributes based on the cell metabolism [25, 26], and in turn kinetic modeling can assist the optimization of medium composition for specified goals.

A well-developed chemically defined medium may roughly contain tens to a hundred components according to the purpose it is used. It typically includes carbohydrates, amino acids, growth factors, lipids, polyamines, trace elements, and vitamins [27, 28]. With the advent of fully chemically defined media, it is now, in theory, possible to explore the impact of each individual medium components on the cell culture performance. The future media development will likely be tailored specific to the cells' characteristics and the desired growth, productivity and product quality

attributes.

Typically, glucose is the main carbon and energy source for mammalian cell culture, meanwhile other carbohydrates such as mannose and galactose may be used as auxiliary sources which has an impact on product quality [22]. A number of amino acids in chemically defined media serve as the main nitrogen source and secondary energy source. In addition to essential amino acids, selected non-essential amino acids are included in chemically defined media depending on the specific cell line and product requirements [9].

Mammalian cells convert these glucose and amino acids into energy, in the form of ATP, and other cellular building blocks through the central metabolic pathways (Fig 1-2). In these pathways, glucose is first converted to pyruvate, which can then either enters the tricarboxylic acid (TCA) cycle for complete oxidation or be channeled toward lactate production. Glutamine and glutamate enters the TCA cycle through the glutaminolysis pathway, while releasing the ammonium groups [29]. Thus, glutamine and glutamate and glucose are the typical three major components that are traced during the cell culture. Which are also considered as major components in cell culture kinetic modeling.

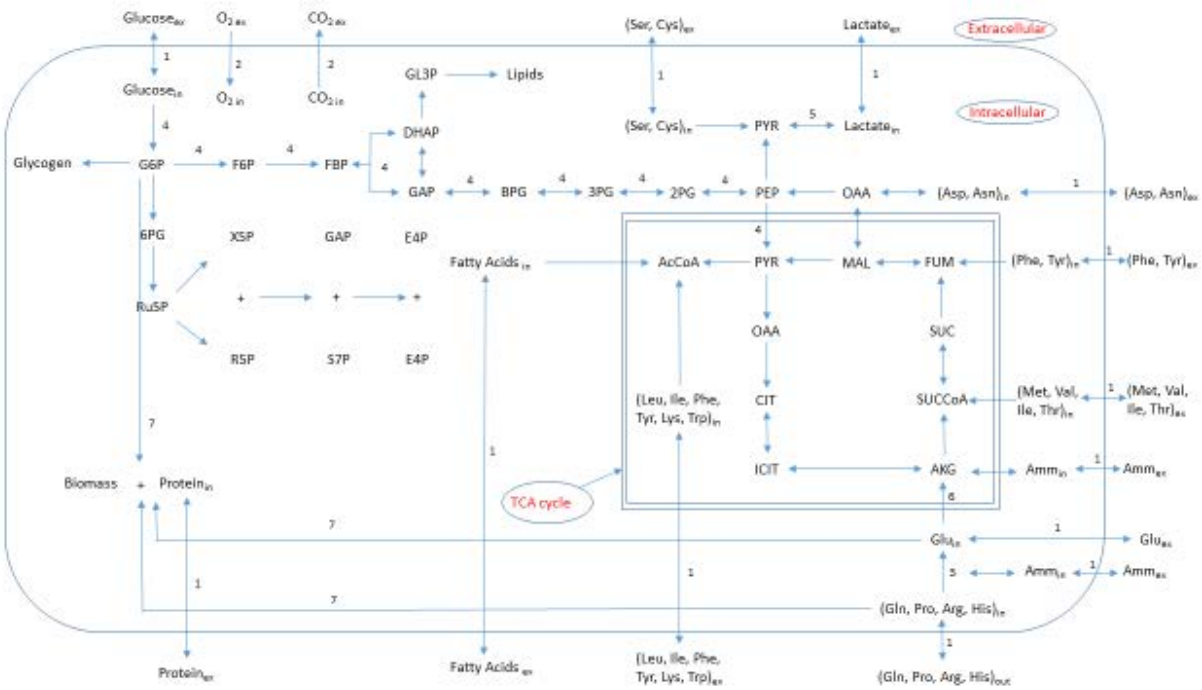


Fig. 1-2. General metabolic processes within CHO cells [30, 31]. The kinetic modeling principles of selected key pathways are shown in Table 1-3.

1.3.3. Bioreactor Modes

Cell culture is conducted in bioreactors which can be operated either in batch, fed-batch or perfusion mode. Batch culture, with no feed being added to the culture, [32] is usually used to obtain information on cell growth and cell stability at the lab scale. Its further application for commercial manufacturing is limited due to the eventual nutrient depletion and the toxic metabolites accumulation.

Fed-batch culture is currently the most prevalent mode for commercial manufacturing. A feed solution containing concentrated nutrients are supplemented to the production culture. This additional nutrients supply can sustain cell growth to higher cell density and maintain production of the recombinant protein for longer duration [33]. A typical fed-batch CHO cell culture can last about two weeks.

Perfusion culture has recently garnered a renewed interest in the industry. In this mode, fresh media replete of nutrients continuously flows into the reactor while perfusate containing the toxic metabolites, product and cells are continuously removed from the reactor at steady state [34]. Thus, the cells remain healthy and the process can be operated continuously. It has reported that the cell culture can last for up to six months [35]. Perfusion culture is particularly preferred for unstable products, such as Factor VIII, which requires short product retention time in the reactor. In certain cases, cells and/or products can be retained in the reactor, which allows a very high cell density and achieves higher protein production than the fed-batch mode [36].

In comparison to traditional stainless steel and glass bioreactors, a disposable bioreactor is a cultivation container which is typically made by FDA-approved polymeric materials and discarded after single usage [37]. Consequently, this reduces the contamination risk and saves the sterilization and cleaning time which is required for stainless steel or glass bioreactors [38]. Meanwhile, disposable equipment allows to turn over the manufacturing suite for different products quickly and save manufacturing plant floor space a lot. Though limitation still exists for disposable bioreactors, such as restricted scalability with a max volume of 2,000-L for most vendors, possible secretion of leachables and extractables, etc. [39, 40], it becomes a good option in the biopharmaceutical industry.

1.3.4. Process parameters

1.3.4.1. Scale dependent parameters

A). Agitation

In bioreactors, agitation is required to maintain nutrients well mixed and disperse

gas. Because CHO cells do not possess cell wall to protect themselves from mechanical damage. They are more sensitive to shear stress than microbes, even with surfactants such as Pluronic-F68 to reduce shear damage [41]. Hence, agitation needs to be carefully chosen to ensure sufficient mass transfer while causing little mechanical damage to the cells. Maintaining constant agitation throughout cell culture is applicable for lab scale bioreactors but may not be suitable for pilot or full scale bioreactors because inhomogeneous mixing or cell damage may occur in large scale bioreactors. Various strategies are used for the optimization of agitation rate in process scale up/down including constant volumetric power, constant impeller tip speed, impeller shear rate and specific impeller pumping rate. More recently, multivariate analysis is used for agitation optimization during scale-up/-down [42, 43].

B). Oxygen/air sparging and CO_2 stripping

Oxygen (O_2) or air is sparged to maintain the necessary sufficient dissolved oxygen (DO), while carbon dioxide (CO_2) stripping is applied to adjust the dissolved CO_2 level and to further control pH during cell culture. However, excessive sparging can damage cells because the cells can attach to the bubbles and get damaged when the bubbles burst.[44] Therefor, similar to agitation, sparging strategy needs to be appropriately developed. O_2 transfer rate and CO_2 transfer rate models based on the two-phase dynamic mass transfer kinetics are usually used for sparging strategy optimization during scale-up/-down [45, 46].

1.3.4.2. Scale independent factors

A). Temperature

Temperature is one of the key process parameters as almost all the enzymatic process within the cells are temperature sensitive. Generally, the optimum temperature

for mammalian cell growth is at around 37 °C [47]. Reported temperature range for CHO cell culture falls between 30 °C to 42 °C [48, 49]. For the purpose of high productivity and biologics quality, temperature may be downshifted during the exponential phase [48, 50-53]. This phenomenon was reported to be correlated with the increased mRNA levels at reduced temperatures and has been examined by metabolic flux analysis [54, 55].

B). pH

A proper pH condition is required to ensure the CHO cells grow and express biologics in a comfortable environment [56]. The media pH usually changes slightly during the cell culture process and is controlled within a near neutral range by Na_2CO_3 and CO_2 [57, 58]. It has also been reported that pH affected cell membrane osmosis due to the Na_2CO_3 added to maintain a neutral extracellular environment [59]. Meanwhile, cell thermo-tolerance and its glycosylation pathway can be sensitive to pH [60, 61]. This means an accurate pH control is essential to achieve good cell culture performance.

1.3.5. Viable cell density and viability

The viable cell density (VCD) and cell viability are two parameters indicative of cell growth and health. It is crucial to grow the cells to a moderate density and maintain the viability throughout the cell culture to achieve high protein titer and quality. Serious cell death and viability drop may lead to the release of toxins and enzymes from the dead cells, which may further lead to a low protein titer or high protein fragmentation. Some cell lines have intrinsically high growth rate and capable of reaching high cell density in the culture, while others have lower growth rate and reach lower cell density.

Meanwhile, appropriate medium can improve cell growth and maintain high viability.

1.3.6. Metabolites

Thousands of metabolites are generated during the cell culture process, among which lactate and ammonia are two well-known toxic metabolites [62-64]. Their accumulation to high levels negatively impacts on the cell health the protein quality [62, 65, 66]. The possible mechanisms for these negative effects are the increase in osmolality, the intracellular and extracellular microenvironment changes as well as some enzymatic processes being affected by the by-product accumulation [67, 68]. Therefore, efficient utilization of nutrients to their overflow to lactate and ammonia is always desired.

During cell culture, it is not unusual to observe that lactate and ammonia are produced at the early stage and then consumed at the late stage [69]. Such metabolic shift from production to consumption usually occurs after the exponential growth phase. It may be because of the intracellular enzyme expression change [31]. Such metabolic shift has often been observed to correlate with higher productivity, though it is currently unknown what exactly triggers it and how to consistently induce it. A number of cell culture feeding strategies and new cell lines have been developed to control lactate and ammonia at low levels or to induce metabolic shift [62, 70, 71].

1.3.7. Protein titer

As the final output, the recombinant protein titer is one key indicator to evaluate a cell culture process. [68, 72, 73]. With the process and facility development in the recent years, the global average commercial mAbs titer from upstream bioprocessing has

increased from 0.20 (g/L) in 1985 to 2.56 (g/L) in 2014, and is expected to reach 3.25 (g/L) in 2024 [74-77]. Table 1 listed the cell culture performance from different cell lines, protein types and bioreactor modes. Though different proteins and CHO cell lines show various kinetic properties, with a well-developed upstream process at fed-batch or perfusion bioreactor mode, μ_G can reach 0.7 (day^{-1}) level with a high productivity μ_P up to 20.1 ($pg\ cell^{-1}\ day^{-1}$), and very high peak cell density, up to 200 × 10^6 (cells/mL) (Table 1) [64]. Some biologics such as bi-specific mAbs and antibody-drug conjugate may have slightly lower titer than the mAbs listed in Table 1-1 [5].

Table 1-1. Relevant bioprocess parameters (peak value) for interferon, Fc fusion protein and other mAbs expression from CHO cells

Therapeutic protein	**Cell lines**	**Peak VCD** (10^6 cells/mL)	μ_P ($pg \cdot cell^{-1} \cdot day^{-1}$)	μ_G (day^{-1})	**Bioreactor mode**	**Year & References**
interferon	CHO-K1	2.6	0.75 – 1.2	0.36 – 0.65	Batch	2010 [78]
	unknown	1.3 – 4.2	0.95 – 3.5	NA		2005 [79]
	CHO-K1	0.35 - 0.8	0.15	0.48	Fed-Batch	2008 [80]
	CHO-K1	3.7	2.6	0.48	Perfusion	2010 [78]
Fc fusion	CHO-K1	> 4	2 - 18	0.6 – 0.7	Fed-Batch	2015 [81]
	CHO-DG44	5.8 - 7.8	9.2 - 20.1	0.51 – 0.66		2014 [64]
mAbs	CHO-DG44	8.5	> 50	0.7	Batch	2015 [8, 9]
	CHO-DG44	23.9	> 50	0.7	Fed-Batch	2015 [9]
	CHO-DUKX	27 – 33.5	> 2	NA	Perfusion	2005 [82]
	unknown	110 - 200	9 - 15	NA		2013 [83]

NA: not available

1.4. Kinetic Modeling

1.4.1. General principles

It is known that numerous dynamic metabolic as well as mass and heat transfer processes occur simultaneously during cell culture [84]. Quite a few cell culture characterizations need to be optimized at limited time to reach good productivity and quality [85]. To speed up the process development, kinetic modeling can be used as a mathematic tool to simulate the cell culture process, analyze and optimize the various process parameters to reduce the time and resources [86].

Conventional kinetic modeling strategies for cell culture heavily depend on intuitional observation and empirical knowledge. These purely empirical or completely unstructured models are regarded as “black box” which is commonly applied for simulating cell culture process in the manufacturing [87]. They are easy to use and can be adapted for data fitting. However, the “black box” kinetic model parameters tell little cell metabolism information, and are prune to fail in optimizing cell culture process.

Thanks to the fast development of the cell metabolic mechanism research and computer technology, more mechanistic modeling strategies have been developed. Though fully mechanistic kinetic modeling strategy which involves thousands of enzymatic processes is still a heavy load even for a super-computer, building up a practical ‘grey box’ strategy can be a meaningful step forward currently. Practical ‘grey box’ models can be developed based on the updated cell metabolic knowledge, cell culture observations and empirical knowledge. As such, they are able to tell some key cell metabolism information, to predict the cell culture performance under extended

conditions, to uncover the possible optimized processes, and to maintain the conventional modeling function of summarizing the large volume of experimental data [87].

Generally, there are four steps in the framework of macroscopic cell culture kinetic modeling (Fig. 1-3) [88]: general mathematic model development, data analysis, model validation and refinery, as well as model based process strategy development. Among all these steps, mathematic model building is the most crucial part, which directly impacts on the accuracy and precision of kinetic modeling.

Step 1 **General Model Development** a) Model structure defined based on cell lines and experiment systems b) Physiological ranges for each kinetic parameter value estimation based on metabolic processes, experiment conditions and literature data
Step 2 **Data Analysis** a) All the required observations in kinetic models collected during the experiment b) Collected data computed with the general model and pre-estimated data c) Kinetic parameters refined within a physiological reasonable range d) Group the “influential” and “non-influential” items
Step 3 **Model Refinery and Validation** a) Models refined with “influential” items b) Models validation with optimized kinetic parameters
Step 4 **Process development** a) Metabolic process based analysis on crucial parameters b) Model predictive capability check with independent dynamic experiments c) Process control strategy optimization and prediction based on validated kinetic analysis

Fig. 1-3. Framework for macroscopic kinetic modeling strategy of CHO cells.

1.4.2. Current kinetic models

Focusing on the important cell culture factors, only key rate-limiting steps deserve kinetic modeling consideration among thousands of intracellular metabolic reactions. Different kinetic models have been applied to describe the cell culture, correlate detectable substrate and metabolite profiles. A summary of CHO cell culture related kinetic model types are listed in Table 1-2. As reported, the Michaelis-Menten (M-M) Equation, Hill Equation, Ordered Binding, Monod-Wyman-Changeux Model (MWC) [89, 90] and Mechanistic Kinetic Model, etc. are the popular models reported and applied [91, 92].

The M-M Equation has been the preferred modeling methods in many cell culture and enzyme kinetic studies due to its convenience for calculation [93-95]. This method simplifies the cell unit as a mono-enzyme, which catalyze the nutrients to the products. It is applicable at a single substrate limiting condition for illustrating cell growth. However, when multiple metabolic pathways become limiting, M-M Equation is not adequate. A comprehensive format of M-M Equation involving multiple substrates and inhibitors was reported as an improved approach [85, 96], though significant deviation can be observed at wide nutrient ranges [97].

Table 1-2. Kinetic models applied in CHO cell culture processes

Type	**Formula**	**Parameter number**	**Ref.**
Monod Equation	$r = \frac{kES}{K + S}$	2	[13]
Hill Equation	$r = \frac{kES^{n_{Hill}}}{K + S^{n_{Hill}}}$	3	[98]
MWC model	$r = ES\left(\frac{\frac{k_T}{k_R K_R}\left(1+\frac{S}{K_R}\right)^{n-1} + \frac{\alpha K_e}{K_R}\left(1+\frac{\alpha S}{K_R}\right)^{n-1}}{\left(1+\frac{S}{K_R}\right)^{n} + K_e\left(1+\alpha\frac{S}{K_R}\right)^{n}}\right)$	5	[5, 99]
Morpheein Model	$r = \frac{k_1 f_1 ES}{K_1 + S} + \frac{k_2(1-f_1)ES}{K_2 + S}$	5	[98]
Random Binding Model	$r = \frac{kEAB}{K_A K_B + K_A B + K_B A + AB}$	3	[31, 100]
Ordered Binding Model	$r = \frac{kEAB}{K_A K_B + K_B A + AB}$	3	[31, 100]
Mechanistic Model	$r = \frac{4kE[S]}{K}\frac{1-\beta+\beta\left(1+\frac{[S]}{\alpha K}\right)^3}{1-\alpha+\alpha\left(1+\frac{[S]}{\alpha K}\right)^4}$	4	[101]
Facilitated Diffusion	$r = \frac{M_o ES_{ex}}{K_d + S_{ex}}$	2	[102]
Passive Diffusion	$r = AD\frac{dS}{dx}$	NA	[102]

By introducing the Hill Coefficient, better simulation results than those using the M-M Equation were reported [103]. However, kinetic parameters lose their mechanistic information due to the forcible introduction of the empirical Hill Coefficient. This

shortcoming makes the Hill Equation only empirically applicable for data manipulation or regression [104].

Binding Models are more mechanistic models than the M-M and Hill Equations. It was derived based on the ordered or random molecule collision occurred intracellularly [102]. This model considered multiple substrates involved in one metabolic pathway. However, for those rate-limiting reactions catalyzed by interactive oligomeric enzymes, it fails to take the enzyme allosteric effect into consideration [105]. Therefore, the validity of simulation results via this model is limited within narrow substrate concentration ranges.

Via taking the interactive properties of key enzymes into consideration, MWC Model and Mechanistic Kinetic Model are able to better simulate the enzymatic pathways and cell culture [90, 98], though it is still at infant stage in applying these two modeling methods with few publication reported. MWC model assumes the enzyme molecules exist as a mixture of two states, exhibiting different ligand binding affinities. Mechanistic kinetic model assumes the enzyme binding affinity and catalytic activity change with different amount of substrate or product ligands binding on the oligomeric enzymes [106]. These two models are more adequate to reflect the nature of intracellular metabolic activities than the above discussed model types. However, they involve more kinetic parameters (Table 2) which means more sampling and analysis work are required to develop the kinetic model.

All the above kinetic models have their advantages and drawbacks, none of them is perfect for all situations. Table 1-3 summarized the reported modeling methods used

for different central metabolic and mass transfer processes illustrated in Fig. 1-1. The selection of modeling strategies based on two principles:

A). Empirical modeling methods are recommended for mass transfer and single substrate-limited processes; while mechanistic modeling methods are more applicable for interactive oligomeric enzymes catalyzed or multiple rate-limiting reactions involved processes.

B). Empirical modeling methods with less kinetic parameters are recommended for a fast operation condition screening and a rapid view of the cell line or process properties; while mechanistic modeling methods with more kinetic parameters are preferred for adequate optimization of the cell culture parameters to achieve excellent performance as well as for trouble shooting to understand the metabolic mechanism.

To get a desired results as well as to balance the time and resource, the utilization of modeling methods should well take the above conditions into well consideration.

Table 1-3. Metabolic kinetics of selected pathways

Metabolic process	**Main Enzyme**	**Reported Model Formula**	**Modeling principle**
Mass transfer		$r = \frac{M_o[E_0]C_{ex}}{K_d + C_{ex}}$	Facilitated Diffusion [102]
		$r = AD\frac{dC}{dx}$	Passive Diffusion [102]
Amide hydrolysis	Glutaminase, glutamine synthetase	$r = \frac{(k_1[Ami] - k_2[GLU][Amm])[E_0]}{1 + K_{Ami}[Ami] + K_{Glu}[GLU] + K_{Amm}[Amm] + K_{Glu}K_{Amm}[GLU][Amm]}$	Random binding [84]
		$r = -(\frac{\frac{k_{T_1}[GLU]}{k_{R_1}K_{R_1}}(1+\frac{[GLU]}{K_{R_1}})^{n_1-1} + \alpha_1 K_{e_1}\frac{[GLU]}{K_{R_1}}(1+\alpha_1\frac{[GLU]}{K_{R_1}})^{n_1-1}}{(1+\frac{[GLU]}{K_{R_1}})^{n_1} + K_{e_1}(1+\alpha_1\frac{[GLU]}{K_{R_1}})^{n_1}}$ $+\frac{\frac{k_{T_2}[Ami]}{k_{R_2}K_{R_2}}(1+\frac{[Ami]}{K_{R_2}}) + \alpha_2 K_{e_2}\frac{[PYR]}{K_{R_2}}(1+\alpha_2\frac{[PYR]}{K_{R_2}})}{(1+\frac{[PYR]}{K_{R_2}})^2 + K_{e_2}(1+\alpha_2\frac{[PYR]}{K_{R_2}})^2})[E_0]$	Monod-Wyman-Changeux Model [107-109]
Glycolysis	Hexokinase, phosphoglucose isomerase, phosphofructokinase, glucose-6-phosphate dehydrogenase, etc.	$r = \frac{k[E_0][GLC]}{K_m + [GLC](1+(\frac{[GLC]}{K_i})^4)}$	Monod Equation Based [110]
		$r = \frac{k[E_0][GLC]}{(1+(\frac{[LAC]}{K_1})^n)(1+\frac{[GLC]}{K_2})}$	Monod Equation Based [84]
		$r = \frac{\mu_G}{YF_{Glc}}X$	Cell culture substrate utilization efficiency [111]
Lactate generation	Lactate dehydrogenase	$r = -(\frac{k_1[LAC]}{1+(\frac{[LAC]}{K_{m_1}})^{n_1}} - \frac{k_2[PYR]}{1+(\frac{[PYR]}{K_{m_2}})^{n_2}})[E_0]$	Hill Equation [112]
		$r = -\frac{V_{\max_1}[E_0]}{1+\frac{K_{m_1}}{[LAC]}+\frac{K_{m_2}}{[NADH]}+\frac{K_{m_1}K_{m_2}}{[LAC][NADH]}}$ $+\frac{V_{\max_2}[E_0]}{1+\frac{K_{m_3}}{[PYR]}+\frac{K_{m_4}}{[NAD^+]}+\frac{K_{m_3}K_{m_4}}{[PYR][NAD^+]}}$	Random Binding [113]
		$r = -(\frac{\frac{k_{T_1}[LAC]}{k_{R_1}K_{R_1}}(1+\frac{[LAC]}{K_{R_1}})^3 + \alpha_1 K_{e_1}\frac{[LAC]}{K_{R_1}}(1+\alpha_1\frac{[LAC]}{K_{R_1}})^3}{(1+\frac{[LAC]}{K_{R_1}})^4 + K_{e_1}(1+\alpha_1\frac{[LAC]}{K_{R_1}})^4}$ $+\frac{\frac{k_{T_2}[PYR]}{k_{R_2}K_{R_2}}(1+\frac{[PYR]}{K_{R_2}})^3 + \alpha_2 K_{e_2}\frac{[PYR]}{K_{R_2}}(1+\alpha_2\frac{[PYR]}{K_{R_2}})^3}{(1+\frac{[PYR]}{K_{R_2}})^4 + K_{e_2}(1+\alpha_2\frac{[PYR]}{K_{R_2}})^4})[E_0]$	Monod-Wyman-Changeux Model [107]

Glycosylation		$r_{Gly} = \frac{k_{cat} C_{E_0} C_p C_m}{K_p K_m + K_p C_m + K_m C_p + C_p C_m}$ [a]	Random Binding [114]
		$r_{Gly} = \frac{k_{cat} C_{E_0} C_p C_m}{K_p K_m (1 + \sum_{i=1}^{NI} \frac{C_{I_i}}{K_{I_i}} + \sum_{j=1}^{NJ} \frac{C_{J_j}}{K_{I_j}}) + K_p C_m (1 + \sum_{i=1}^{NI} \frac{C_{I_i}}{K_{I_i}}) + K_m C_p (1 + \sum_{j=1}^{NJ} \frac{C_{I_j}}{K_{I_j}}) + C_p C_m}$ [b]	
		$r_{Gly} = \frac{k_{cat} C_{E_0} C_p C_m}{(K_p K_m + K_p C_m + K_m C_p + C_p C_m)(1 + \sum_{h=1}^{N} \frac{C_{I_h}}{K_{I_h}})}$ [c]	
		$r_{Gly} = \frac{k_{cat} C_{E_0} C_p C_m}{K_p K_m + K_p C_m + C_p C_m}$ [a]	Ordered Binding [114]
		$r_{Gly} = \frac{k_{cat} C_{E_0} C_p C_m}{K_p K_m (1 + \sum_{i=1}^{NI} \frac{C_{I_i}}{K_{I_i}}) + K_m C_p (1 + \sum_{j=1}^{NJ} \frac{C_{I_j}}{K_{I_j}}) + C_p C_m}$ [b]	
		$r_{Gly} = \frac{k_{cat} C_{E_0} C_p C_m}{(K_p K_m + K_m C_p + C_p C_m)(1 + \sum_{h=1}^{N} \frac{C_{I_h}}{K_{I_h}})}$ [c]	
		$r_{Gly} = \frac{k_{cat} C_{E_0} C_p C_m}{K_p C_m + K_m C_p + C_p C_m}$ [a]	Ping-Pong Binding [114]
		$r_{Gly} = \frac{k_{cat} C_{E_0} C_p C_m}{K_p C_m (1 + \sum_{i=1}^{NI} \frac{C_{I_i}}{K_{I_i}}) + K_m C_p (1 + \sum_{j=1}^{NJ} \frac{C_{I_j}}{K_{I_j}}) + C_p C_m}$ [b]	
		$r_{Gly} = \frac{k_{cat} C_{E_0} C_p C_m}{(K_p C_m + K_m C_p + C_p C_m)(1 + \sum_{h=1}^{N} \frac{C_{I_h}}{K_{I_h}})}$ [c]	

[a], [b] and [c] indicate the equations are applicable for no inhibitor, competitive inhibitors and non-competitive inhibitors situations, respectively.

1.4.3. Kinetic properties of CHO cell culture

Table 1-1 presents a literature example of CHO cell culture at different conditions. The kinetic characteristics are influenced by the properties of cell lines, cell culture processes and therapeutic proteins. As discussed above, perfusion mode always allows

a high peak VCD and higher productivity, though the manufacturing cost is also higher than the conventional batch and fed-batch modes. Meanwhile, when expressing mAbs, the cell growth and protein titer were observed higher than that when expressing Fc fusion and interferon.

1.4.4. Scale-up modeling

Scale-up/-down is an integral part of cell culture. Scale-up bridges the lab-scale knowledge to manufacturing [43, 48]. Scale-down is important for simulating the manufacturing scale performance at lab-scale for research, trouble shooting and process development purposes in order to save time and cost [42]. In a pilot or full scale bioreactor, the scale independent parameters can be controlled relatively coincident as that in a lab scale bioreactor, while the scale dependent parameters need to be appropriately developed.

Successful translation of a cell culture process across different scales require careful consideration of scale up/down strategies. In general, physical conditions in a large bioreactor cannot exactly duplicate those in a small bioreactor although geometric similarity is maintained [102]. Several phenomena are unique to large scale which are not typically observed at lab scale such as limitations on rates of agitation and CO_2 stripping. Various models are currently available to evaluate the effect of scale and to guide scale-up/-down strategies. A summary of the scaling criteria for suspension cell cultures at similar geometric bioreactors are listed in Table 1-4. While volumetric energy input and impeller top rate are more applied for microbial process development, CHO cell culture scale-up/down is much based on volumetric mass transfer constant

and oxygen or carbon dioxide transfer rate.

Table 1-4. Summary of scaling criteria for suspension cell cultures [43] [102, 115]

Scaling criteria	Notation
Volumetric energy input	$P/V = \rho N_p \omega^3 D^5 / V$
Tip velocity	ωD
Specific impeller pumping rate	$Q/V \propto \omega$
Volumetric mass transfer constant	$k_L a \propto \omega^{\alpha} q^{\beta}$ or $k_L a \propto (P/V)^{\alpha} (Q_G / S)^{\beta}$
Reynolds Number	$\mathrm{Re} = \omega D^2 \rho / \mu$
Oxygen uptake rate	$OUR = OTR = k_L a (C_L^* - C_L)$ at equilibrium
Mixing Time	$t_{mix} \propto \frac{V^a \mathrm{Re}^b}{D}$
dCO_2 removal rate	$R_{dCO_2} = \frac{10^{-pH}}{10^{-pH} + K_C} k_L a_{CO_2} (C_{CO_2}^* - C_{CO_2})$

Where, P_0 is the impeller power; ω is the impeller rotation number; D is the impeller diameter; V is the working volume; N_P is the impeller power number; $k_L a$ is the volumetric mass transfer constant; q is the sparing rate; α, β and a, b are the empirical coefficients; Q_G is the gas volumetric rate; S is the tank cross-sectional area; R_e is the Reynolds Number; ρ is the medium density; μ is the medium viscosity; C_L^* and C_L are equilibrate and dynamic dissolved oxygen concentration, respectively; $C_{CO_2}^*$ and C_{CO_2} are equilibrate and dynamic dissolved carbon dioxide concentration, respectively.

1.5. Case Studies-medium optimization

As discussed above, medium design is one crucial task for cell culture. Among all the nutrients, carbohydrates are the main carbon and energy source which should be well optimized. Numerous researches on this topic have been carried out in the recent decades [22, 63, 116, 117], while few of them are well analyzed with kinetic modeling

method. With appropriate modeling, new knowledge can be extracted from researches reported even 18 years ago [22], which was confirmed after 6 and 10 years of the original publication via other independent researches [70, 71].

In this chapter, the experimental data are taken from Altamirano et al. (2000) [22], who reported also semi-quantitative comparison results. In the experiment, CHO-TF70R cell culture was cultivated for t-PA production. The culture was carried out with 7 mM amino acids and 20 mM monosaccharides, a stirring rate of 50 rpm with 96% relative humidity in an atmosphere of 5% CO_2 in air. The cell culture was conducted in spinner flasks in a CO_2 incubator at 37 °C. To get the kinetic information, the original experimental data were reanalyzed with the modeling strategies listed in Table 1-3. The simulation was carried out with the Excel Visual Basic program ODExLims. The calculated kinetic characteristics are shown in Table 1-5.

Table 1-5.Selected values of the cell culture kinetic characterizations for CHO-TF70R cell line growing on different carbohydrates and amino acids sources.

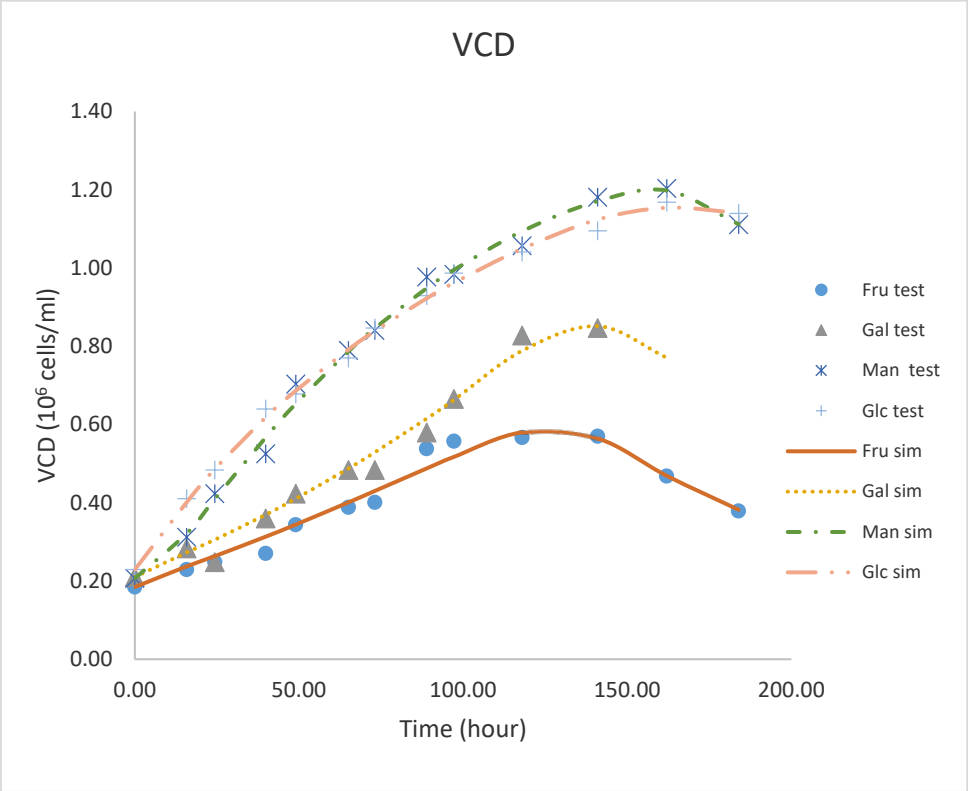
Amino acids	**Glutamine**				**Glutamate**	
Monosaccharides	**Fructose**	**Mannose**	**Glucose**	**Galactose**	**Glucose**	**Galactose**
μ_G (10^{-3} h^{-1})	0~13	2.6~31	2.2~26	0~13	8.6~27	7.2~21
μ_d (10^{-3} h^{-1})	0~9.4	0.2~12	0~3.1	0~4.6	3.1~8.5	2.9~7.7
μ_P (ng/(h·10^6 cells))	0~59	1.2~56	0~48	0~57	12~46	8.5~71

The cell was cultivated with glutamine and four different monosaccharides. The cell culture processes were well simulated (Fig. 1-4). It shows that mannose and glucose could achieve the highest peak while fructose had the poorest performance. The outstanding effect from mannose and glucose was confirmed with the large μ_G shown in Table 5. However, the computed μ_G of galactose and fructose are similar. The higher peak VCD from galactose than fructose was due to the smaller μ_d. Comparing the cell growth properties (μ_G and μ_d) of all these four monosaccharides on cells, mannose and glucose provided the high cell growth ability, while glucose and galactose also inhibited cell death the most. Thus, glucose was the best choice for cell growth.

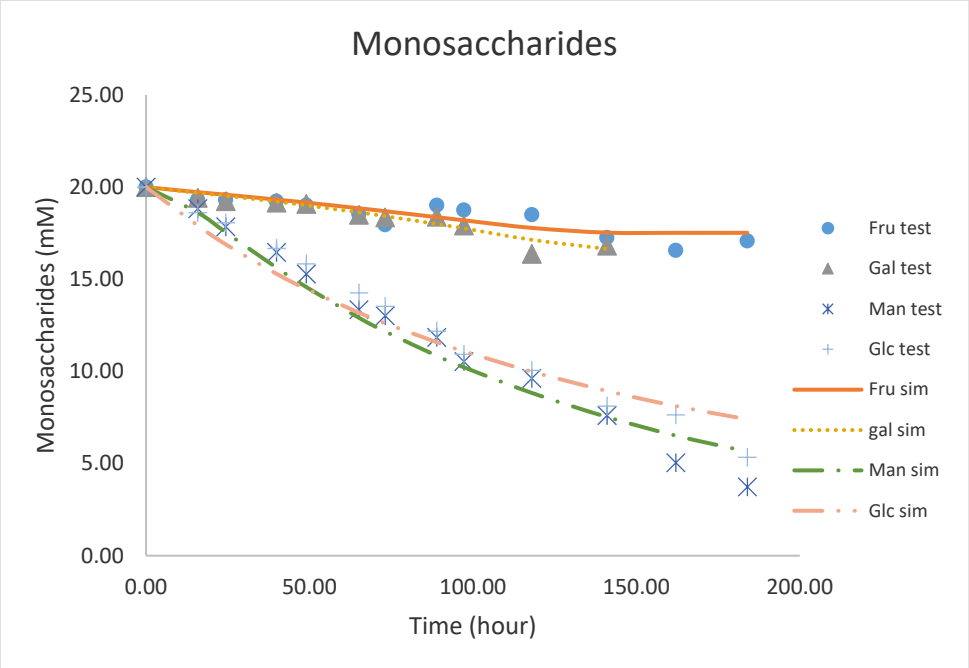
Shown in Fig. 1-4, mannose achieved the highest protein titer, while a computed results of μ_p (Table 1-5) shown that mannose, fructose and galactose actually presented similar protein production ability from each cell, slightly higher than glucose. This points to a possible combination of two monosaccharides in the media at different

stages to achieve high protein titer. For example, it may be applicable to use a high glucose ratio media for biomass accumulation in the exponential phase and feed in high galactose, fructose or mannose ratio medium when the cells enter therapeutic protein expression stage. This strategy was later proposed in 2006 [71] and 2011 [70] by other independent researchers, though it could be calculated from the Altamirano's experiments in 2000 via kinetic modeling [22].

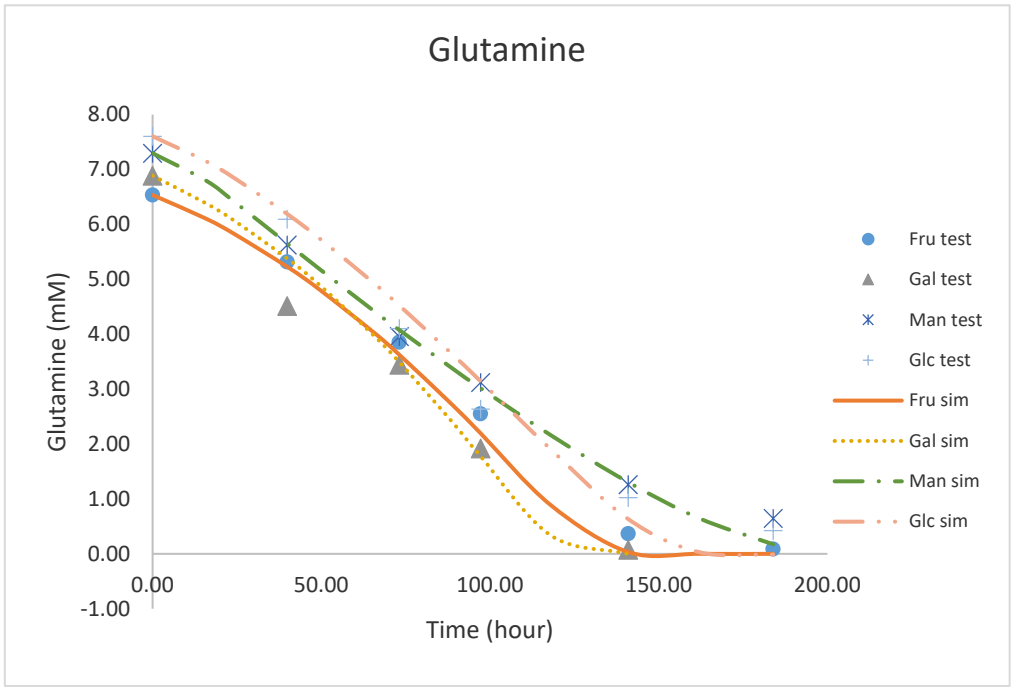
(A)


VCD
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
VCD (10⁶ cells/ml)
0.00
50.00
100.00
150.00
200.00
Time (hour)
Fru test
Gal test
Man test
Glc test
Fru sim
Gal sim
Man sim
Glc sim

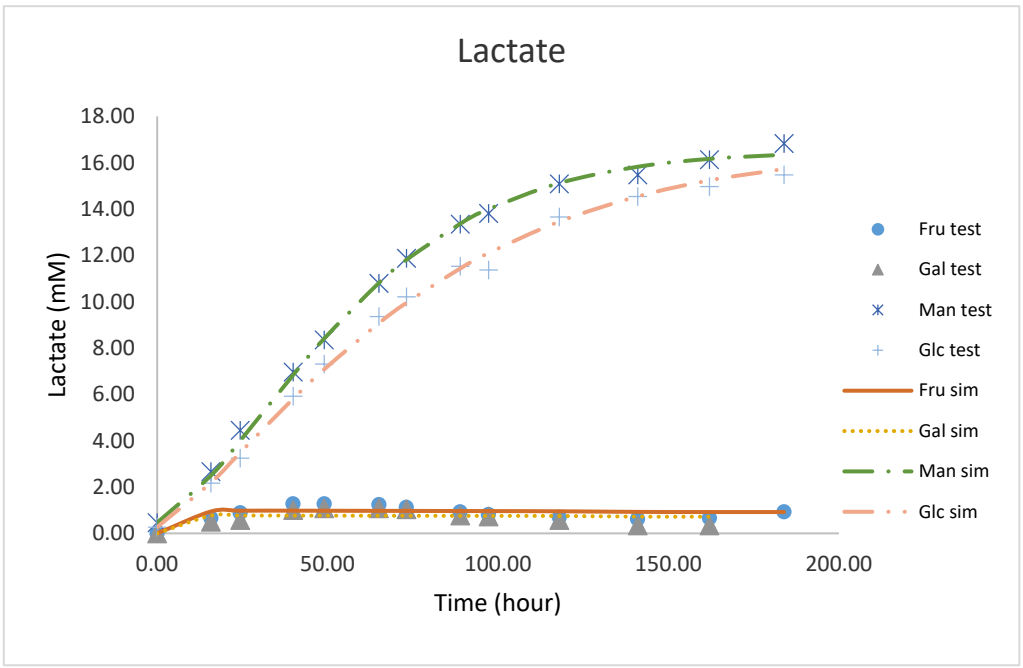
(B)


Monosaccharides
25.00
20.00
15.00
10.00
5.00
0.00
Monosaccharides (mM)
0.00
50.00
100.00
150.00
200.00
Time (hour)
Fru test
Gal test
Man test
Glc test
Fru sim
gal sim
Man sim
Glc sim

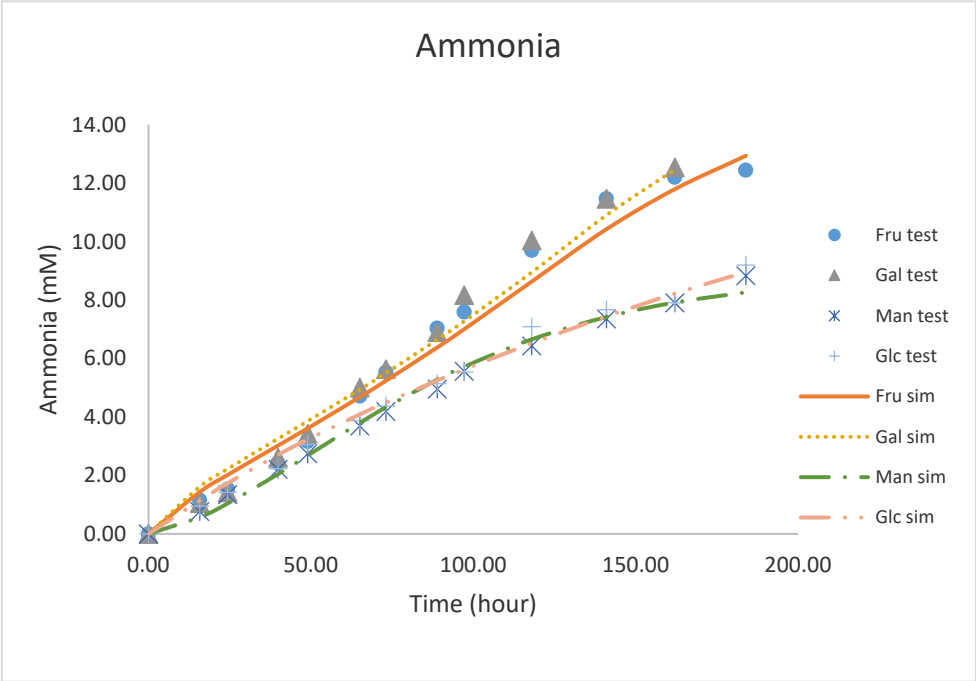
(C)


Glutamine
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
Glutamine (mM)
0.00
50.00
100.00
150.00
200.00
Time (hour)
Fru test
Gal test
Man test
Glc test
Fru sim
Gal sim
Man sim
Glc sim

(D)


Lactate
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Lactate (mM)
0.00
50.00
100.00
150.00
200.00
Time (hour)
Fru test
Gal test
Man test
Glc test
Fru sim
Gal sim
Man sim
Glc sim

(E)



(F)

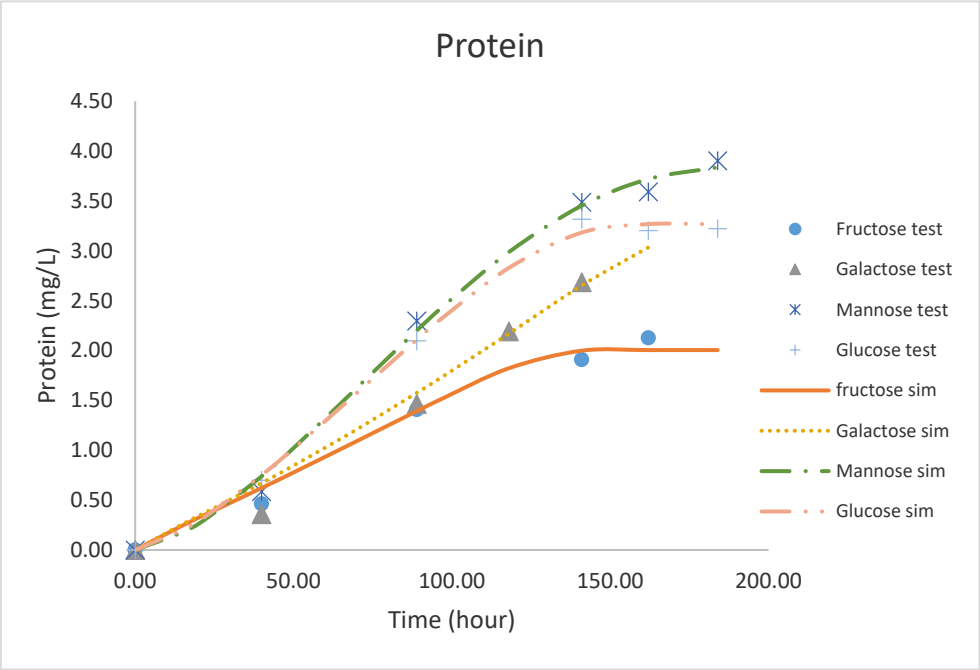


Fig. 1-4. Profiles of CHO-TF70R cell culture for t-PA production with different carbohydrates: (A) VCD; (B) Monosaccharides; (C) Glutamine; (D) Lactate; (E) Ammonia; (F) Protein. The points are the experimental data and the lines are the simulation results.

1.6. Challenges and perspectives of kinetic modeling for CHO cell culture upstream process development

Despite the rapid development of CHO cell kinetic modeling in the recent years, the models keep updating with new discoveries from mammalian cell metabolic and genetic engineering researches. Thus, it is challenging to set up standard models that can be used for one decade or applicable for most of the current research projects with different purposes. Theoretically, kinetic modeling is able to elucidate the mechanism of the protein quality attributes. However, a better understanding of the quality attribute mechanism may be required to build up the models. Meanwhile, with the application of perfusion and single-use technology, it is necessary to validate the applicability of the kinetic characteristics from the stainless steel batch or fed-batch bioreactors.

In order to better serve cell culture, further improvement of kinetic modeling may combine it with other technologies to form a powerful platform methodology. For example, metabolic flux analysis can be used for dynamic metabolic information detection.[118] This detail information can help to understand the metabolic mechanism and build up models to elucidate the quality attributes. Also, the utilization of powerful supercomputer makes it possible to compute more kinetic processes within reasonable time. This may allow rapid on-line kinetic analysis in the future, which can be a strong tool for process automation, monitoring and fast metabolic disorder diagnosis for process development and manufacturing.

1.7. Conclusion

In this work, crucial cell culture factors were reviewed and kinetic modeling principles and strategies were systematically summarized. One case study was presented to give an examples of medium optimization via kinetic modeling. Though kinetic modeling is not a current obligatory part for documentation by FDA, it is definitely beneficial for data analysis, process prediction, trouble shooting, and scale-up/-down. With the awareness of its importance and the continuous development of kinetic modeling, it is potential to be an efficient and practical tool for the process development and manufacturing in the near future. Future cell culture kinetic modeling research may combine with other technologies to form a standard and powerful platform tool to better serve the biopharmaceutical industry.

While this chapter provides a general introduction of kinetic modeling principles, the following chapter will focus on the discussion of current biopharmaceutical industry. It helps to know the importance and the potential applicable market of this study.

Chapter 2

Screening of Monoclonal Antibodies for Cancer Treatment

2.1.Abstract

The global biologics market is growing rapidly. The screening process of suitable biologics and indications is important to design appropriate pre-clinical, clinical and marketing strategies. A general definition of 'screening' in the biopharmaceutical industry includes three aspects: the appropriate biologics for the specific cancers, the appropriate indications for the specific biologics, and the promising biologic candidates from the pools at the pre-clinical drug discovery stage. Effective screening strategies in the biopharmaceutical industry are crucial to accelerate the drug commercialization process and select the effective biologics for patients. The current status of commercial mAbs and the global pharmaceutical market was briefly summarized in this chapter. The mechanism of commercial mAbs and the indications, as well as the current technologies for mAbs screening in the new drug discovery and cell line development stages were systematically reviewed. This work aims at being a beneficial reference for screening high-quality mAbs, appropriate indications with efficient technologies.

Keywords: screening, cancer treatment, biomarker, monoclonal antibodies.

2.2.Introduction

Cancer is a global leading cause of death [119]. It is featured as unregulated cell division and growth [120]. Caused by genic mutation or gene expression disorder,

abnormal metabolism can be observed within cells [121]. While gene therapy is still away from well accepted by FDA, monoclonal antibodies (mAbs), as one of the major parts of biologics, are currently widely recognized drugs for conservative cancer treatment. The mAbs are antibodies made by identical cells, which are all derived from a unique parent immune cell [122, 123]. The history of mAbs can be traced back to 1975 when recombinant DNA technology was applied to antibody design [124]. The first mAb approved by FDA was OKT3 in 1986 [125], though it took almost three decades to the current 'golden age' of cancer therapies using mAbs [126]. Now, nearly a hundred commercial mAbs are available in the global market while hundreds of candidates are in the clinical trials.

The biopharmaceutical industry is regarded, as a matter of fact, a high risk and high revenue industry. On average, it takes $1.2 to $ 4 billion and 10 to 12 years for one biologic candidate to be approved and enter the market from the very beginning. From the risk point of view, less than 0.1 % of the biologic candidates before CMC stages are able to enter into Phase I. Among those biologics, 60 % fail to pass Phase II, while there's another 50% failure risk at the clinical stage Phase III. In addition, there are significantly higher risks at earlier new drug discovery stages. Thus, an efficient and successful screening of mAb candidates and corresponding indications is crucial. The word "screening" in the biopharmaceutical industry refers to three aspects:

A). Screening of drug or biologic candidates for specific diseases;

B). Screening the potential indications for specific drugs or biologics; and

C). Screening of promising drug or biologic candidates from the pool in the pre-clinical

stage.

Undoubtedly, all the aspects are definitive for the destiny of one biologic candidate. In this chapter, the current commercial mAbs and the recognized biomarkers were systematically reviewed. The principles, criteria, modeling and detection methods of biologics screening were presented and compared. It aims at providing comprehensive screening information for cancer treatment, which is potentially beneficial for research institutes, pharmaceutical companies, and patients.

2.3.Mechanism

It is known that one distinctive characteristics of cancer from other diseases is that immune cells have difficulties to distinguish tumor cells from normal cells [123]. Therefore, a process that can either assist the immune cells to identify the tumor cells, or stimulate the immune cells to be more active is potential for cancer treatment. MAbs, which are designed for distinguishing the biomarkers specifically expressed by tumor or immune cells, are recognized as promising biologics. Though the exact metabolic details of how mAbs work is awaiting better understanding, the general mechanism typically falls into two categories:

A). mAbs distinguish and bind the biomarkers abnormally expressed by the tumor cells, helping the immune cells or the drugs to target these cells. For example, trastuzumab, which was designed to target the biomarkers HER2, is a representative commercial mAb for breast cancer.

B). The immune cells are activated by mAbs to attack the tumor cells. Successful

commercialized examples, such as nivolumab and pembrolizumab which target PD-1 and PD-L1 biomarkers; respectively, were designed based on such a mechanism.

With the development of cancer mechanism understanding, hundreds of biomarkers have been detected, and some of them have been used for mAbs design. Table 2-1 listed 47 recognized biomarkers that have been successfully used for commercial mAbs. Indications that relate to the abnormal expression of these biomarkers have been well studied. This table aims to help narrow down the screening scope of the mAbs and indications, as well as predict the clinical results and control the quality of the designed protein therapeutics, which is in compliance with the Quality by Design (QbD) principles. This information may help biopharmaceutical industry to make decisions on biologics design at early discovery stage or on indication selection at early clinical stages.

Table 2-1. The recognized biomarkers for biologics and the approved indications

Antigen Biomarkers	Indications	Ref.
α-4 integrin	Multiple sclerosis	[127]
BLyS	Systemic lupus erythematosus	[128]
CCR4	Relapsed or refractory adult T-cell leukemia/lymphoma	[129]
CD3	Transplant rejection, organ	[130]
CD6	Psoriasis, Arthritis, rheumatoid	[131]
CD19	Precursor B-cell acute lymphoblastic leukemia	[132]
CD20	Relapsed or refractory low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma, chronic lymphocytic leukemia, and multiple sclerosis	[133]
CD30	Hodgkin lymphoma, and anaplastic large-cell lymphoma	[134, 135]
CD38	Multiple myeloma	[136]
CD52	B-cell chronic lymphocytic leukemia	[137]
Clostridium difficile toxin B	Prevent recurrence of Clostridium difficile infection	[138]
Complement component 5	Paroxysmal nocturnal hemoglobinuria	[139]
CTLA-4	Metastatic melanoma	[140]
Dabigatran	Emergency reversal of anticoagulant dabigatran	[141]
EGFR	Metastatic colorectal carcinoma, and metastatic squamous non-small cell lung carcinoma	[142, 143]
EpCAM	Malignant ascites, multiple cancers	[144]
F protein of RSV	Respiratory syncytial virus	[145]
Ganglionside P3	Multiple cancers	[146]
GD2	Pediatric high-risk neuroblastoma	[147]
GPIIb/IIIa	Percutaneous coronary intervention	[148]
HER2	Metastatic breast cancer	[149]
IgE	Moderate to severe persistent asthma	[150]
IL12	Plaque psoriasis	[151]
IL23	Psoriatic arthritis, plaque psoriasis, and crohn's disease	[151, 152]
IL17A / IL17RA	Plaque psoriasis	[153]
IL1B	Cryopyrin-associated periodic syndrome	[154]
IL2R	Multiple sclerosis	[155]
IL2RA	Prophylaxis of acute organ rejection in renal transplant	[156]
IL4RA	Atopic dermatitis	[157]
IL5	Severe asthma	[158]

IL6	Multicentric Castleman's disease	[159]
IL6R	Rheumatoid arthritis, and systemic juvenile idiopathic arthritis	[160]
IL8	Psoriasis	[161]
integrin receptor	Ulcerative colitis, crohn's disease	[162]
PCSK9	Heterozygous familial hypercholesterolemia, and refractory hypercholesterolemia	[163]
PD-1	Metastatic melanoma, and metastatic squamous non-small cell lung carcinoma	[164-166]
PD-L1	Urothelial carcinoma, metastatic non-small cell lung cancer, and metastatic Merkel cell carcinoma	[167, 168]
PDGFRA	Soft tissue sarcoma	[169]
Protective antigen of Bacillus anthracis / Anthrax toxin	Inhalational anthrax	[170]
PSMA	Diagnostic imaging agent in newly diagnosed prostate cancer or post-prostatectomy	[171]
RANKL	Postmenopausal women with osteoporosis	[172]
SLAMF7	Multiple myeloma	[173]
TNF	Rheumatoid arthritis, juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, crohn's disease, ulcerative colitis, and plaque psoriasis	[174, 175]
TNF α	Crohn's disease	[174]
VEGF	Metastatic colorectal cancer	[176, 177]
VEGFR1	Wet age-related macular degeneration	[178]
VEGFR2	Wet age-related macular degeneration, and gastric cancer	[177, 179]

2.4.Design Technologies Selection

Unlike small molecule drugs that can directly enter cells and interrupt the metabolism with poor capacity of discernment, mAbs are mild and impact indirectly on the metabolic pathways with excellent targeting ability on tumor cells. To improve the efficacy as well as avoid patent disputes, several advanced design technologies have been applied to improve mAb performances. The characteristics of different biologic design technologies are briefly summarized in Table 2-2, which were further discussed

as follows.

Table 2-2. Different biologic design technologies with their characteristics

Design Technology	Characteristics			
	Manufacturing Cost	Expression (g/L)	Purification Recovery (%)	Efficacy
Conventional	Low	2 – 10	60 – 80	Normal
ADC	Medium	0.4 – 5	50 - 70	Small & Large Molecule
Bi-specific	Medium	0.5 – 3	<20	Tumor & Immune Cells (Synergistic)
Combined Medication	High	Depends	Depends	Synergistic Effect

2.4.1. Antibody Drug Conjugate

Antibody drug conjugate (ADC) technology is one improvement strategy, which allows small molecules conjugated on an antibody molecule [180]. Via the excellent targeting ability of the protein therapeutics, the small molecule is able to directly interrupt the metabolic pathways of tumor cells with significantly improved capacity of discernment [181]. Nevertheless, challenges that ADC faces in manufacturing include:

1. Unstable expression level due to the variety of the link structure, especially, when the link consists of unnatural amino acids;
2. Low downstream yield due to the added purification steps after the conjugation of the small molecule drug to the protein molecule.

2.4.2. Bi-specific

Bi-specific antibody technology is another option for better biologics design. It allows one biologic molecule to recognize two biomarkers simultaneously.

Theoretically, the combination of the two biomarkers should fall into one of the three types: both on tumor cells, both on immune cells, and one on tumor cell and one on immune cell. Nevertheless, the third design is the most preferred in the industry. By this design, the immune cells can be effectively activated, then rapidly and adequately attack the tumor cells in situ [182]. This makes the protein therapeutics exhibit synergistic effect compared to using two or more independent mAbs. This technology has the potential to enhance the efficacy while reducing the biologics dosage and side effects. However, bi-specific molecules also have their drawbacks in manufacturing, such as:

1. Low expression level during cell culture due to the high risks of chain mispairing and protein aggregation;
2. Low downstream separation efficiency because of the similar physicochemical properties of the mispairing molecules.

2.4.3. Combined Medication

Combined medication technology is an alternative choice to improve the efficacy. The joint usage of two or more biologics, or biologics with small molecule drugs has the potential to exhibit synergistic effect on tumor cells, because different biologics and/or drugs may impact on different metabolic pathways. This technology provides one strategy to screen new indications for existing mAbs avoiding huge investment for new drug design and application. This strategy, however, has some disadvantages for commercialization, including:

1. Significantly high cost in manufacturing, factory operation, storage, and supply chain management for different products/molecules;

2. Difficulties in maintaining acceptable stability of different biologics and/or drugs if in one formulation;
3. Inconvenience and high cost in drug delivery if different formulations were used for different biologics/drugs.

2.5.Commercial mAbs

With increasing attention being focused on mAbs, a large amount of investment has been attracted into the biopharmaceutical industry for biologics commercialization. Fig. 2-1 lists the top 10 best-selling mAbs in 2016 and their global annual sales from 2014 to 2016. The data were obtained from the annual reports of these enterprises. Little rank change was observed in Fig. 2-1, except Opdivo was regarded as a dark-horse in the recent years due to the excellent performance of this anti-PD-1 mAb. Humira, Remicade, and Rituxan kept the top three best-selling for the recent three years (Fig. 2-1), bringing considerable revenue to Abbvie, Johnson & Johnson, and Roche, respectively. In 2016, the total sales of these ten mAbs were $ 61.2 billion, which is almost 70% of the whole global antibody market. The global oligopolistic market landscape may not be broken in the next few years due to limited number of validated biomarkers and long period of time for one biologic product to be commercialized.

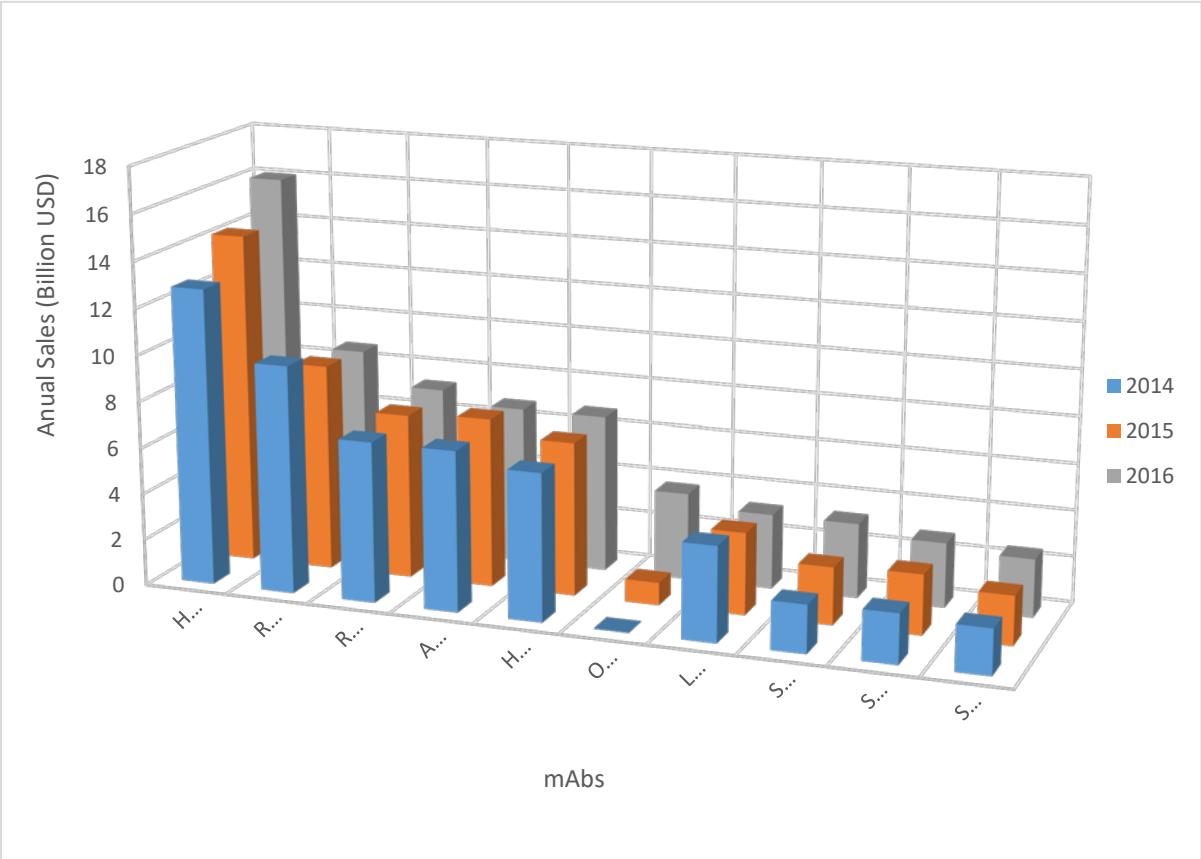


Fig. 2-1 Top 10 best-selling mAbs in 2016 and their global annual sales in the recent three years. Different colors refer to the selling data in different years.

A list of biomarkers with corresponding commercial mAbs and patent holders were shown in Table 2-3. The mAbs and patent information was filtered manually from an open source tool called '*Citeline Service*' before July 16th, 2017. Most of the biosimilars currently in research were designed based on these listed mAbs. This table also includes the major biomarkers for ADC and bi-specific antibodies, which is a trend for biologic design in the next few years.

Table 2-3. Commercial biologics and the corresponding antigen biomarkers, patent holders and the biologic license application (BLA) approved dates

Antigen Biomarkers	Biologics	Patent Holders	BLA Approved
α-4 integrin	Tysabri	Biogen Idec	11/23/2004
BLyS	Benlysta	Human Genome Sciences	03/09/2011
CCR4	Poteligeo	Amgen	03/30/2012, Japan
CD6	Alzumab	Center of Molecular Immunology	01/07/2013, India
CD19	Blincyto	Amgen	12/03/2014
CD20	Zevalin	Spectrum Pharmaceuticals	02/19/2002
	Gazyva	Genentech	11/01/2013
	Ocrevus		03/28/2017
	Rituxan		10/26/2009
	Arzerra	Glaxo Grp	11/26/1997
CD30	Adcentris	Seattle Genetics	09/19/2011
CD38	Darzalex	Janssen Biotech	11/16/2015
CD52	Campath	Genzyme	05/07/2001
	Lemtrada		
Clostridium difficile toxin B	Zinplava	Merck	10/21/2016
Complement component 5	Soliris	Alexion	03/16/2007
CTLA-4	Yervoy	Bristol-Myers Squibb	03/25/2011
dabigatran	Praxbind	Boehringer Ingelheim	10/16/2015
EGFR	Erbitux	ImClone Systems	02/12/2004
	Portrazza	Eli Lilly	11/24/2015
	Vectibix	Amgen	09/27/2006
	BIOMAB EGFR	Biocon	11/12/2007, India
	Theraloc	Oncoscience	11/12/2003, EMEA
	CIMAher	Center of Molecular Immunology	11/18/1994, Cuba
CD3	Ior-t3[a]	Center of Molecular Immunology	05/15/1996, Cuba
	Removab	Fresenius, Swedish Orphan Biovitrum, Neovii Biotech	01/27/2011, EMEA
EpCAM			
F protein of RSV	Synagis	Med-Immune	06/19/1998
Ganglionside P3	Vaxira	Recombio, Laboratorio Elea,	12/31/2012, Cuba

		Innogene Kalbiotech	
GD2	Unituxin	United Therapeutics	03/10/2015
GPIIb/IIIa	ReoPro	Centocor	12/22/1994
HER2	Kadcyla	Genentech	02/22/2013
	Perjeta		06/08/2012
	Herceptin		09/25/1998
IgE	Xolair		06/20/2003
IL12 IL23	Stelara	Centocor	09/25/2009
		Janssen Biotech	09/23/2016
IL17A	Taltz	Eli Lilly	03/22/2016
	Cosentyx	Novartis	01/21/2015
IL17RA	Siliq	Valeant	02/15/2017
IL1B	Ilaris	Novartis	06/17/2009
IL2R	Zinbryta	Biogen	05/27/2016
IL2RA	Simulect	Novartis	05/12/1998
	Zenapax	Roche	12/10/1997
IL4RA	Dupixent	Regeneron	03/28/2017
IL5	Nucala	GlaxoSmithKline	11/04/2015
	Cinqair	Teva	03/23/2016
IL6	Sylvant	Janssen Biotech	04/23/2014
IL6R	Actemra	Genentech	01/08/2010
			10/21/2013
	Kevzara	Sanofi	02/01/2017, Canada
IL8	ABCream[a]	Yes Biotech	07/13/2004, China
integrin receptor	Entyvio	Takeda	05/20/2014
PCSK9	Praluent	Sanofi Aventis	07/24/2015
	Repatha	Amgen	08/27/2015
PD-1	Opdivo	Bristol-Myers Squibb	12/22/2014
			03/04/2015
	Keytruda	Merck	09/04/2014
PD-L1	Tecentriq	Genentech	05/18/2016
			10/18/2016
	Bavencio	EMD Serono	03/23/2017
	Imfinzi	AstraZeneca	05/01/2017
PDGFRA	Lartruvo	Eli Lilly	10/19/2016
Protective antigen of Bacillus anthracis	Raxibacumab	Human Genome Sciences	12/24/2012
Protective antigen of the Anthrax toxin	Anthem	Elusys Therapeutics	03/18/2016
PSMA	ProstaScint	Cytogen	10/28/1996
RANKL	Prolia	Amgen	06/01/2010

	Xgeva		
SLAMF7	Empliciti	Bristol-Myers Squibb	11/30/2015
TNF	Humira	Abbvie	12/31/2002
	Amjevita	Amgen	09/23/2016
	Cimzia	UCB (company)	04/22/2008
	Simponi	Centocor	04/24/2009
	Simponi Aria	Janssen Biotech	07/18/2013
	Renflexis	Samsung Bioepis	04/21/2017
	Inflectra	Celltrion Healthcare	04/05/2016
TNF α	Remicade	Centocor	08/24/1998
VEGF	Avastin	Genentech	02/26/2004
VEGFR1	Lucentis		06/30/2006
VEGFR2			
	Cyramza	Eli Lilly	04/21/2014

Note: "[a]" refers that it is the generic name of the biologics instead of the commercial name.
If the BLA was not approved by FDA, the biologics was specified with its approved location or organization.

2.6.mAbs Screening Methods

2.6.1. Screening criteria

After the designing of the mAbs based on the mechanisms discussed above, there could still be thousands of candidates available. This will be followed by two major screening processes to obtain best performed antigen-specific antibodies from the pool, which are:

1. Binding screening including specificity [183] & affinity [184];
2. Functional screening including cell growth, proliferation, apoptosis, endothelial tube formation, etc. [185]

While the functional assays are based on different disease models, the binding screening assays are universal in biopharmaceutical industry.

Specificity is the ability of the antibody binding to its cognate antigen and not to other targets. Affinity is the characteristic of antibody-antigen binding strength. These two criteria are crucial to ensure the efficacy, while good specificity can minimize the side-effects and good affinity is well preferred to reduce the drug dosage.

Functional activities are often the most significant characteristics of an antibody, including ability to deliver a toxin, antagonist activity, partial and full agonist activity, etc. These activities are often related to the protein allostery via the antigen-antibody specific binding [186, 187].

2.6.2. Screening Models

To quantitatively evaluate the above criteria, kinetic modeling strategies are usually applied [188]. Known models include Michaelis–Menten (M–M) model [189], Hill Equation [94, 190-192], different types of Binding Models [105, 193, 194], Morpheein Model, Monod–Wyman–Changeux (MWC) model [99], Mechanistic kinetic description strategy [101], and empirical models derived from software such as JMP [195]. The advantages and drawbacks of each modeling strategy have been discussed in chapter 1.

Table 2-4 presented a summary of the above models with their typical mathematic formula and applicable scopes. In the current biopharmaceutical researches, M-M Model and Hill Equation are the favored modeling strategies for biologics screening, due to their simplicity. While Morpheein Model, MWC Model and Mechanistic kinetic description strategy are able to well describe the kinetic properties of the molecular interactions. They are preferred when the kinetic mechanism understanding is critical

to explain the biologics behavior. Different types of Binding Models can be applied for multiple molecules involved reactions, such as bi-specific antibody involved reactions. Different strategies are selectively used based on the study purposes and research limitations.

Table 2-4. Reported kinetic modeling strategies for molecular interaction study.

Type	Formula	Applicable Scope	Ref.
M-M Model	$r = \frac{kES}{K+S}$	non-interactive oligomeric or mono-molecular interactions	[189, 193]
Hill Equation	$r = \frac{kES^{n_{Hill}}}{K+S^{n_{Hill}}}$	Data manipulation for all molecular interactions	[91, 192]
MWC model	$r = ES\left(\frac{\frac{k_T}{k_R K_R}\left(1+\frac{S}{K_R}\right)^{n-1} + \frac{\alpha K_e}{K_R}\left(1+\frac{\alpha S}{K_R}\right)^{n-1}}{\left(1+\frac{S}{K_R}\right)^{n} + K_e\left(1+\alpha\frac{S}{K_R}\right)^{n}}\right)$	Oligomeric molecular interactions	[196, 197]
Morpheein Model	$r = \frac{k_1 f_1 ES}{K_1+S} + \frac{k_2(1-f_1)ES}{K_2+S}$	Oligomeric molecular interactions	[98]
Random Binding Model	$r = \frac{kEAB}{K_A K_B + K_A B + K_B A + AB}$	Three molecule involved interactions	[194, 198]
Ordered Binding Model	$r = \frac{kEAB}{K_A K_B + K_B A + AB}$	Three molecule involved interactions	[194, 199]
Mechanistic Kinetic Description	$r = \frac{4kE[S]}{K}\frac{1-\beta+\beta\left(1+\frac{[S]}{\alpha K}\right)^{n-1}}{1-\alpha+\alpha\left(1+\frac{[S]}{\alpha K}\right)^{n}}$	Oligomeric molecular interactions	[98, 101]

2.6.3. Binding Screening Assays

Enzyme-linked immunosorbent assay (ELISA) is one of the most popular platform technologies to identify antigen-antibody complex and both qualitatively and quantitatively evaluate the binding strength. The basic principle of ELISA based on

radioimmunoassay techniques dates back top 1941 [200], and the exact method was created in 1971 [201]. Currently, it is a major detection method for biologics screening, because it is simple, quick, sensitive, specific, and high-throughput [202]. Another screening technology is surface plasmon resonance (SPR) biosensor [203]. As a gold standard for real-time and label-free monitoring technology of biomolecular interactions, it is able to determine the thermodynamic and kinetic properties of specific molecular interactions [204].

While ELISA and SPR are the common techniques for extracellular or cell-free antigen-antibody binding detection, fluorometric microvolume assay (FMAT) and fluorescence-activated cell sorting (FACS) are well-developed methods for on-cell or native binding screening [205]. The working principle based on antibodies binding to the antigen expressed on cell surface, and the immunoglobulin constant region of the antibodies is detected by a fluorescently conjugated secondary antibody. As a high-throughput cell-based assay in the hybridoma screening, FMAT and FACS based technologies significantly improved the screening efficiency and success probability.

2.7.Conclusion

Though cancers are not incurable disease due to the rapid technology development, they are still a leading threat for human health. In this chapter, the recent trends and technologies of mAb development are comprehensively summarized. The information of biomarkers, indications, commercial mAbs and the pattern status were systematically reviewed. It is beneficial for biopharmaceutical industry, research institutes and patients

to make decisions. It aims at providing a comprehensive understanding of the biomarker, indication and mAb screening strategies. This may promote further advancements in new drug discovery, novel indications of exiting drugs, as well as joint usage of mAbs and other cancer treatment methods.

Based on the above two chapters, the kinetic modeling knowledge and the biopharmaceutical industry have been introduced. The following chapter will give a case study of the kinetic modeling application for cell culture process development to reach a high titer.

Chapter 3

Development of Temperature Shift Strategies for Chinese Hamster Ovary Cell Culture Based on Kinetic Modeling

3.1.Abstract

Improving cell growth and titer of recombinant protein is crucial in biomanufacturing. To achieve this goal, temperature shift (TS) strategies are widely used. Though some studies have been published regarding the temperature's effect on Chinese hamster ovary (CHO) cell culture, there is no systematic method of TS strategy development available. In this work, the temperature impact was systematically studied using two industrial CHO cell lines for production of two model monoclonal antibodies. Short-duration experiments with kinetic modeling were applied for rapid TS strategy development. Kinetic parameters derived from the resulting experimental data were subsequently used to compute the cell culture performance at extended periods. This study aims at developing an efficient method for systematic CHO cell bioprocess TS optimization to reach a high titer and appropriate cell growth, while providing an understanding of temperature's effect on cell culture performance and quality attributes.

Keywords: Chinese hamster ovary cell culture; kinetic modeling; monoclonal antibody; temperature shift; quality attributes; titer

3.2.Introduction

Chinese Hamster Ovary (CHO) cell lines are prevailing hosts for therapeutic protein production in the biopharmaceutical industry [3, 4]. Temperature control, as an important bioprocess factor [206], remains a topic of great interest for CHO cell culture [48, 50-53, 207, 208]. Temperature shift (TS) to a hypothermic condition during the exponential growth phase is one favorable strategy to balance cell growth, protein expression, and quality [209-211]. Such a strategy holds the potential to improve cell culture performance for a prolonged period [207]. However, few models to design TS strategies have been reported [212]. Furthermore, current methods of TS strategy development require substantial resource investment into laboratory experiments, which heavily relies on non-systematic application of empirical knowledge. This presents a challenge because it requires exhaustive experimental investigation to identify the optimum TS strategy. Therefore, an efficient method to quantitatively evaluate temperature effect and to systematically screen TS strategies is beneficial for time sensitive process development in the biopharmaceutical industry.

Kinetic modeling provides a mathematic method to simulate, analyze, and predict cell culture processes [96, 102, 213]. Structured models which mechanistically contain complete cell culture metabolism information are able to explicitly illustrate the cell metabolism [96] and the enzymatic reactions [98]. However the complexity of intracellular processes as well as the involvement of hundreds of chemicals and reactions makes it difficult to apply this class of model in CHO cell culture [214]. Therefore, unstructured models, such as Monod type relationships are widely used to

describe CHO cell culture [96, 215-219]. Unstructured models partially characterize cell culture properties utilizing measurements such as cell density, nutrients, and metabolites while neglecting complex intracellular metabolism. The parameters can be used to indicate the activities of considered metabolic pathways, and utilized to evaluate the cell culture performance at extended conditions.

In this study, batch and fed-batch CHO cell culture experiments were conducted to estimate kinetic parameters. It is concluded that before the exhaustion of nutrients, batch and fed batch modes give similar cell culture performance characteristics due to similar intracellular metabolism, except higher cell density and volumetric titer observed from the fed batch culture. Thus we assumed that little metabolism variation occurs with slight changes in conditions, the kinetic pattern should remain relatively unchanged in describing the cell culture bioprocess at reasonable extended time durations and different TS strategies [220]. This assumption will hold true when the two scenarios exhibit the same complete integral patterns from cell growth to cell death and therapeutic protein expression. It allows us to employ short duration ($\leq$ 8 days) cell culture experiments starting from a high seeding density to approximate TS strategies of a full period (14 days) cell culture starting from a normal density.

The objective of this chapter is to develop an efficient method to understand the temperature effect on CHO cell culture and provide a systematic strategy to develop TS strategies with short duration experiment for high therapeutic protein productivity via kinetic modeling. It aims at reducing the bioprocess development time, cost and associated laboratory work in the biopharmaceutical industry.

3.3.Methods

3.3.1. Experiments and materials

3.3.1.1.Cell line and cell culture

CHO cell line A (CHO-A) was cultured in 250-ml shake flasks with 80-ml initial working volume and proprietary chemically defined basal media for monoclonal antibody production. Shake flasks were initiated with a 10×10^6 cells/mL inoculation density and cultured at constant temperatures of 32, 33, 34, 35 and 36.5 °C for 8 days. Meanwhile, a 0.6×10^6 cells/mL inoculation density was cultured at 6 different TS strategies for 14 days. Shaking rate and CO_2 were controlled at 150 rpm and at 6% saturation, respectively. Daily feeding started when cells reached an appropriate VCD. Additional glucose was supplied to maintain a proprietary concentration range [221].

CHO cell line B (CHO-B) was cultured in 5-L Sartorius glass bioreactors with Finesse control systems at 3.5 L initial working volume and proprietary chemically defined basal media. Experiments were initiated with a 0.6×10^6 cells/mL inoculation density at constant culture temperatures of 33, 34, 35, 36.5, 37.5, and 38.5 °C for 4 days. Agitation was fixed at 240 rpm, and the pH and DO were controlled at pH 7.1 and 50% saturation, respectively.

To study the temperature effect on quality attributes, CHO-B was also cultured in 2-day batch shake flasks with shaking rate at 150 rpm and incubator CO_2 was maintained at 6% saturation. A high inoculation density of 10×10^6 cells/mL was applied at constant temperatures of 33, 34, 35, 36.5, 37.5, and 38.5 °C.

All the above experiments were conducted in duplicates.

3.3.1.2.Cell culture assays

VCD and cell viability were quantified off-line using a Vi-CELL XR automatic cell counter (Beckman Coulter). Glucose, glutamine, glutamate, lactate, and ammonia were quantified with a CEDEX Bio HT analyzer (Roche). A Protein A HPLC method was used to measure protein titer [221]. The highest protein titer was set as 100 % normalized titer in this study. For bioreactor samples, off-line pH, pCO_2 and pO_2 were detected using a Bioprofile pHOx analyzer (Nova Biomedical). The measured valued were larger than 3 × noise signal and reported less than 5 % difference in the duplicated experiments.

3.3.1.3.Protein quality attribute assays

Size exclusion chromatography (SEC) was performed using a Tosoh TSK G3000SW_{xl} column, 7.8 cm × 30 cm × 5 μm, with an isocratic gradient monitored at 280 nm on a Waters Alliance HPLC system (Milford, MA) equipped with a temperature controlled autosampler and Waters 2996 PDA detector.

Charge Variants were assayed by Imaged Capillary Isoelectric Focusing (iCIEF), which was performed on a Protein Simple iCE3 instrument with an Alcott 720 NV autosampler (San Jose, CA). Samples were mixed with appropriate pI markers, ampholytes, and urea and injected into a fluorocarbon coated capillary cartridge. A high voltage was applied and the charged variants migrated to their respective pI. A UV camera captured the image at 280 nm. The main peak was identified and the peaks that migrated into the acidic range and basic range were summed, quantitated, and reported as relative percent area.

N-Glycans analysis was performed using a commercially available kit from Prozyme, GlykoPrep® Rapid N-Glycan Preparation with 2-AB (Hayward, CA). The free oligosaccharides were profiled using an Acquity UPLC Glycan BEH Amide, 130 Å, 1.7 µm × 2.1 mm × 10 mm column (Milford, MA) on a Waters Acquity H-Class system (Milford, MA) equipped with a temperature controlled autosampler and fluorescence detector.

3.3.2. Model Development

Intracellular metabolism involves hundreds of chemicals and enzymatic reactions, many of which are not mechanistically understood [214, 222]. It is therefore not trivial to take all these reactions into consideration. Thus, an unstructured kinetic approach will be used to focus on nutrients, such as carbohydrates and amino acids, which contribute to biomass accumulation, and toxic metabolites that disturb the desired metabolic pathways. In this study, glucose, glutamine and glutamate were regarded as the representative nutrients, while lactate and ammonia were regarded as the representative toxic metabolites [96]. Thus, Monod based models including the main influential detected substrates and metabolites are applied to describe cell growth [111, 223].

$$r_X = (\mu_G - \mu_d)X \tag{3.1-a}$$

$$\mu_G = \mu_{\max} \prod_{i=1} \frac{[S_i]}{KI_{S_i} + [S_i]} \prod_{j=1} \frac{KI_{P_j}}{KI_{P_j} + [P_j]} \tag{3.1-b}$$

$$\mu_d = k_d \prod_{i=1} \frac{KD_{S_i}}{KD_{S_i} + [S_i]} \prod_{j=1} \frac{[P_j]}{KI_{P_j} + [P_j]} \tag{3.1-c}$$

Where, r_X is the cell growth rate; μ_G is the specific growth rate; μ_d is the specific death rate; μ_{max} is the maximum specific cell growth rate; k_d is the maximum specific cell death rate; X is the viable cell density (VCD), S is the concentration of the limiting substrate, P is the concentration of toxic metabolites; KI_S, KI_P, KD_S and KD_P are the saturation constants.

Glucose is the main carbon and energy source, following the kinetic process described by Xing et al. [96]

$$r_{Glc} = -\left(a_{Glc} + \frac{\mu_G}{YF_{Glc}} \right) X \tag{3.2-a}$$

Where, r_{Glc} is the glucose concentration change rate; YF_{Glc} is glucose consumption coefficient; $\frac{\mu_G}{YF_{Glc}}$ indicates the glucose consumption associated with cell growth; a_{Glc} denotes the glucose consumption to maintain cell viability. Equation (3.2-a) works for the batch mode.

Considering the intermittent feeding of glucose during a fed-batch mode, an integral formation of equation (3.2-a) should be written as

$$\int_{t_1}^{t_2} r_{Glc} = \sum_{i=1}^{n} C_{Glc-i} + \int_{t_1}^{t_2} -\left(a_{Glc} + \frac{\mu_G}{YF_{Glc}} \right) X \tag{3.2-b}$$

Where, two adjacent measurement are taken at t_1 and t_2; $C_{Glc\text{-}i}$ is the concentration of feeding glucose; $\sum_{i=1}^{n} C_{Glc-i}$ indicates the total glucose added into the bioreactor.

Though glutamine and glutamate are included in the media, they can also be generated from other substrates catalyzed by different enzymes, and then entered into

the central metabolism [31]. Their kinetic processes can be expressed as

$$r_{G\ln} = \left(\sum_j \frac{k_{G\ln_i} C_i}{K_{G\ln_i} + C_i} - a_{G\ln} - \frac{\mu_G}{YF_{G\ln}} \right) X \tag{3.3-a}$$

$$r_{Glu} = \left(\sum_j \frac{k_{Glu_j} C_j}{K_{Glu_j} + C_j} - a_{Glu} - \frac{\mu_G}{YF_{Glu}} \right) X \tag{3.4-a}$$

Where the subscript $_{Gln}$ and $_{Glu}$ denote glutamine or glutamate, respectively; C_i and C_j are the i-th chemical that will generate glutamine or glutamate; r_{Gln} and r_{Glu} are the glutamine and glutamate concentration change rates; YF_{Gln} and YF_{Glc} are glutamine and glutamate consumption coefficients; $\frac{\mu_G}{YF_{G\ln}}$ and $\frac{\mu_G}{YF_{Glu}}$ indicate the glutamine and glutamate consumption associated with cell growth; a_{Gln} and a_{Glu} denote the glutamine and glutamate consumption to maintain cell viability; k is rate constant and K is saturation number in Michaelis-Menten model.

With a well-developed chemically defined medium, there are typically sufficient nutrients to supply glutamine and glutamate. Thus, their generation can be regarded approximately stable during the cell culture process, that is,

$$\sum_i \frac{k_i C_i}{K_i + C_i} = b_{A\min o} \tag{3.5}$$

Where, b_{amino} is a constant.

Substituting (3.5) to (3.3-a) and (3.4-a) leads to

$$r_{G\ln} = -\left(d_{G\ln} + \frac{\mu_G}{YF_{G\ln}} \right) X \tag{3.3-b}$$

$$r_{Glu} = -\left(d_{Glu} + \frac{\mu_G}{YF_{Glu}} \right) X \tag{3.4-b}$$

Where d_{Gln} and d_{Glu} are constants that can be either negative or non-negative. Equations (3.3-b) and (3.4-b) work for the batch mode.

Similarly as glucose, when taking consideration of the daily feed of glutamine and glutamate during a fed-batch mode, an integral formation of equations (3.3-b) and (3.4-b) should be written as

$$\int_{t_1}^{t_2} r_{G\ln} = \sum_{i=1}^{n} C_{G\ln-i} + \int_{t_1}^{t_2} -\left(d_{G\ln} + \frac{\mu_G}{YF_{G\ln}}\right)X \tag{3.3-c}$$

$$\int_{t_1}^{t_2} r_{Glu} = \sum_{i=1}^{n} C_{Glu-i} + \int_{t_1}^{t_2} -\left(d_{Glu} + \frac{\mu_G}{YF_{Glu}}\right)X \tag{3.4-c}$$

Where, $C_{Gln\text{-}i}$ and $C_{Glu\text{-}i}$ are the concentrations of glutamine and glutamate concentration daily fed to the bioreactor; $\sum_{i=1}^{n} C_{G\ln-i}$ and $\sum_{i=1}^{n} C_{Glu-i}$ indicate the total glutamine and glutamate added.

As known traceable metabolites, lactate and ammonia have known effects on cells [28, 224]. They are generated from the central metabolism to support cell growth and maintain cell viability, while being consumed during cell culturing [28, 31, 224, 225]. Thus, the process can be expressed as

$$r_{Lac} = \left(a_{Lac} + b_{Lac}\mu_G - \frac{k_{Lac}[Lac]}{K_{Lac} + [Lac]}\right)X \tag{3.6}$$

$$r_{Amm} = \left(a_{Amm} + b_{Amm}\mu_G - \frac{k_{Amm}[Amm]}{K_{Amm} + [Amm]}\right)X \tag{3.7}$$

Where, r_{Lac} and r_{Amm} are the lactate and ammonia concentration change rates; a and b indicate the metabolite produced to maintain cell survival and associated with cell

growth, respectively. The metabolites can be consumed during the cell culture, catalyzed by enzymes such as lactate dehydrogenase or transaminases.

To characterize the therapeutic protein production process, various substrates and toxic metabolites should be considered [22, 28, 226]. Organized in a comprehensive Michaelis-Menten structure, the kinetic equation can be expressed as [114]

$$r_{TP} = Q_{TP} X \tag{3.8-a}$$

$$Q_{TP} = KP \prod_{i=1} \frac{[S_i]}{K_{S_i} + [S_i]} \prod_{j=1} \frac{K_{P_j}}{K_{P_j} + [P_j]} \tag{3.8-b}$$

Where Q_{TP} is the specific therapeutic protein production rate; KP is the maximum specific protein production rate; K_S and K_P are saturation constants.

3.4.Results and Discussion

3.4.1. Variables and constants from modeling

Integral cell culture kinetic information can be extracted from the 8-day high seeding density fed-batch experiments, including cell growth and death, metabolites concentration change, and therapeutic protein production.

Though *KI*, *KD*, and *K* denoting the enzyme binding affinities should theoretically respond to temperature change, our preliminary investigation using the equations (3.1) – (3.8) revealed that these parameters kept relatively steady (<10 % variation) within the temperature range studied. Therefore, the *KI*, *KD*, and *K* values were considered approximately constant in this study. This provided an intuitional comparison of μ_d, *YF*, *k* and *KP* values to describe the temperature effect on cell growth, cell metabolism and

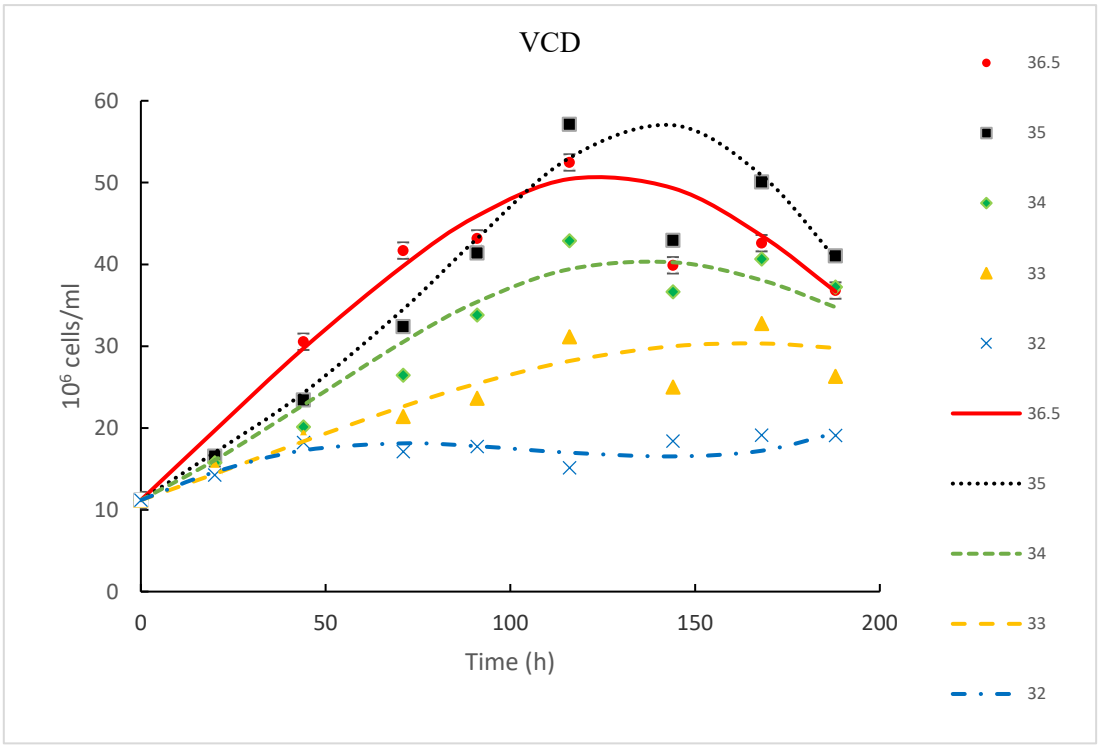
protein productivity. Glucose was not a limiting substrate indicated by a small calculated KI_{Glc} value (~10^{-3}g/L). This agrees with the feeding strategy of sufficient glucose supplement.

The on-line temperature measurement indicated that it took less than 15 minutes to reach the shifted temperature in a 5-L bioreactor. Thus, sudden temperature shifts were assumed for this study.

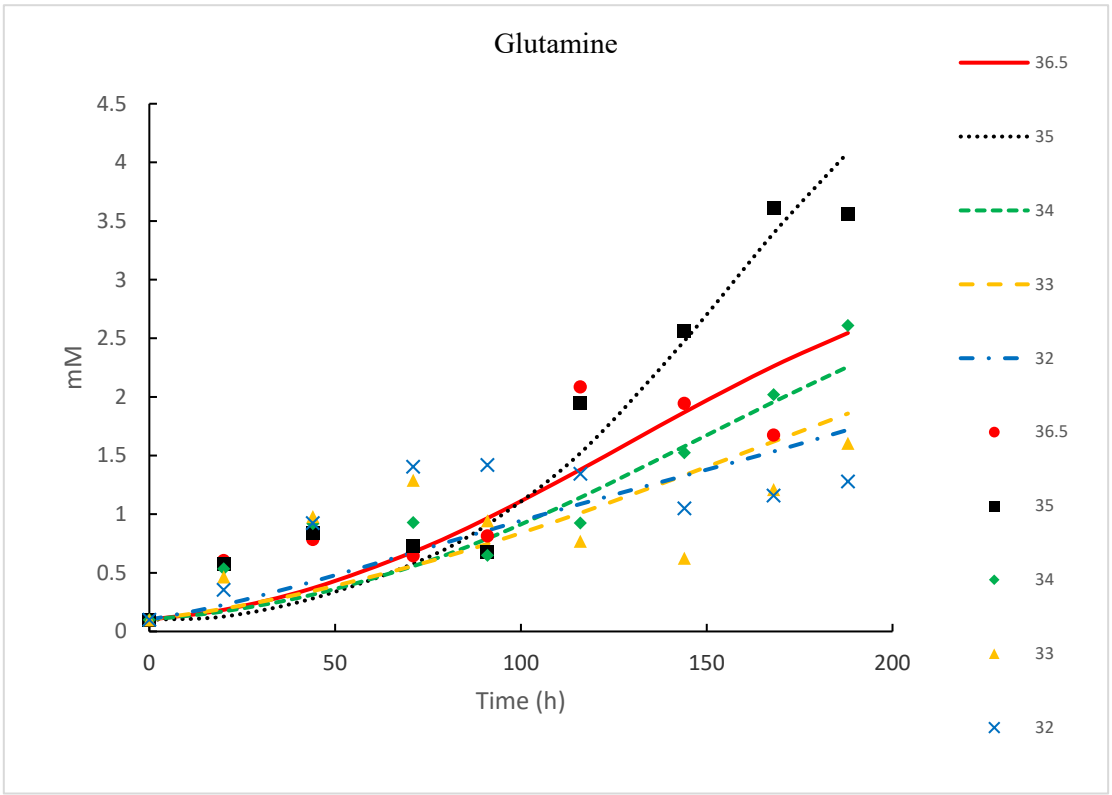
3.4.1.1.CHO-A

At a high seeding density, complete mammalian cell culture patterns can be observed within 8-days (Fig. 3-1). Accelerated cell growth and death rates were associated with increased temperature (Table 3-1), as indicated by μ_{max} (Fig. 3-2 A, Table 3-1) and k_d. This observation supports applying an appropriately high temperature for cell growth and subsequently a TS down to prolong cell viability when VCD was high. Though at 36.5 ºC CHO-A exhibited the highest growth rate in the exponential phase, it reached the highest peak VCD at 35 **ºC**. The μ_{max} indicated that at these two temperatures, CHO-A growth ability was similar (Fig. 3-2 A). The difference of the VCD profiles may be due to their different correlations to the metabolite profiles.

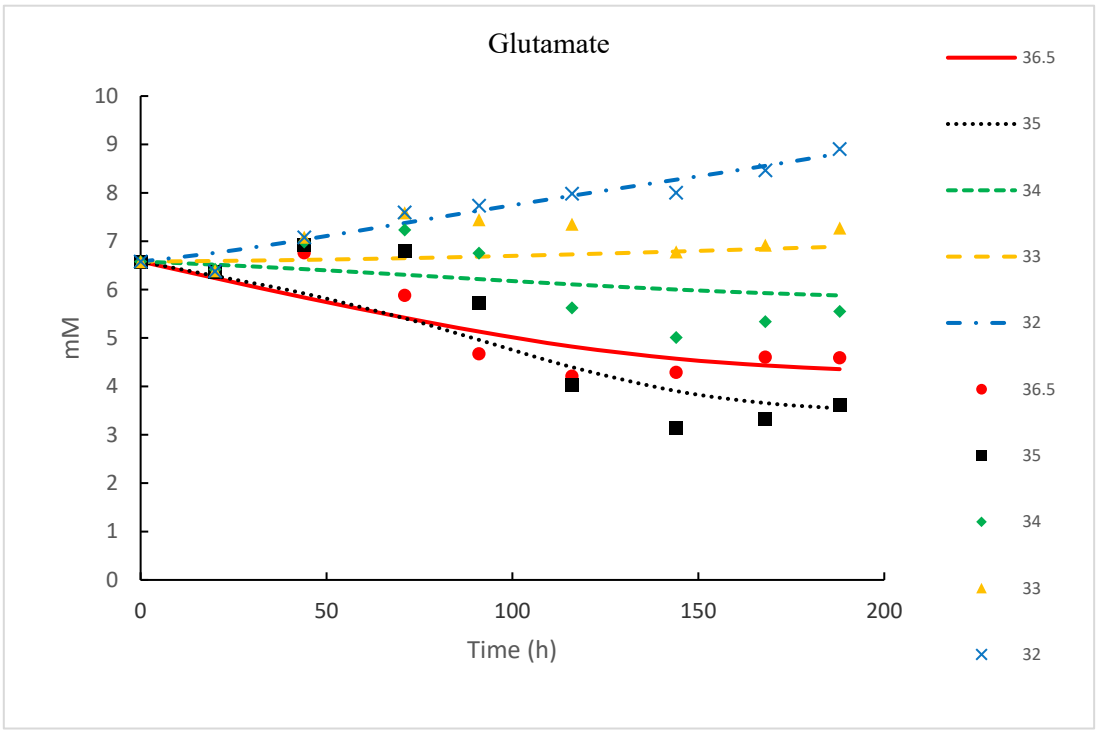
A


VCD
60
50
40
30
20
10
0
10^6 cells/ml
0
50
100
150
200
Time (h)
36.5
35
34
33
32
36.5
35
34
33
32

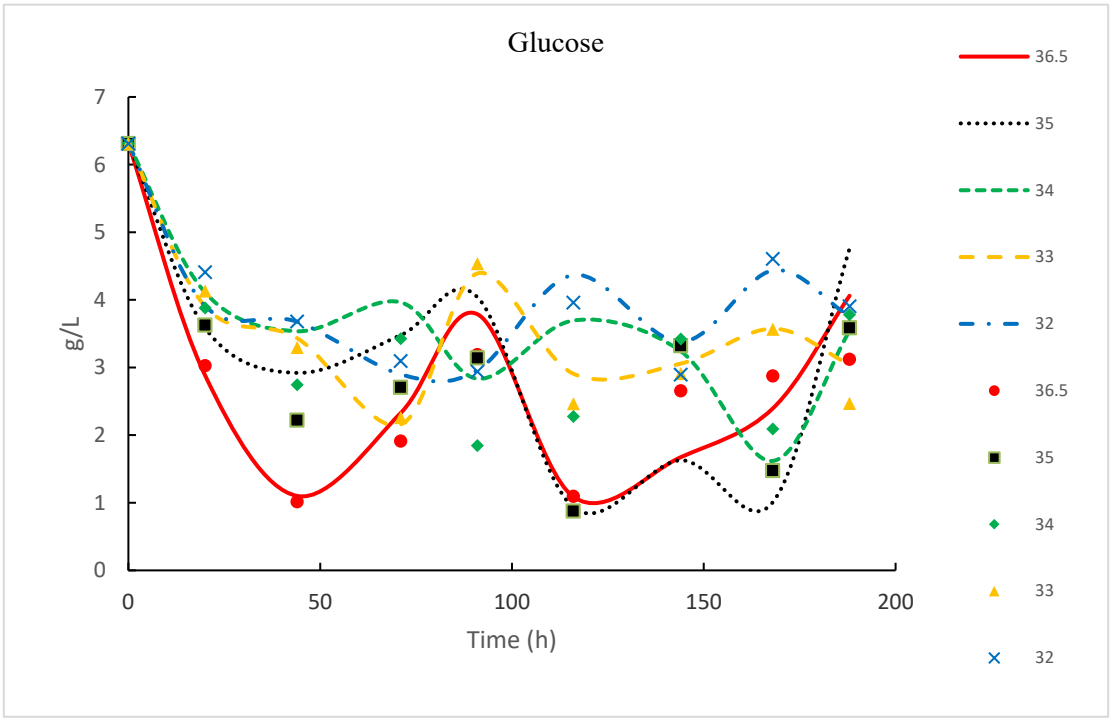
B


Glutamine
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
mM
0
50
100
150
200
Time (h)
36.5
35
34
33
32
36.5
35
34
33
32

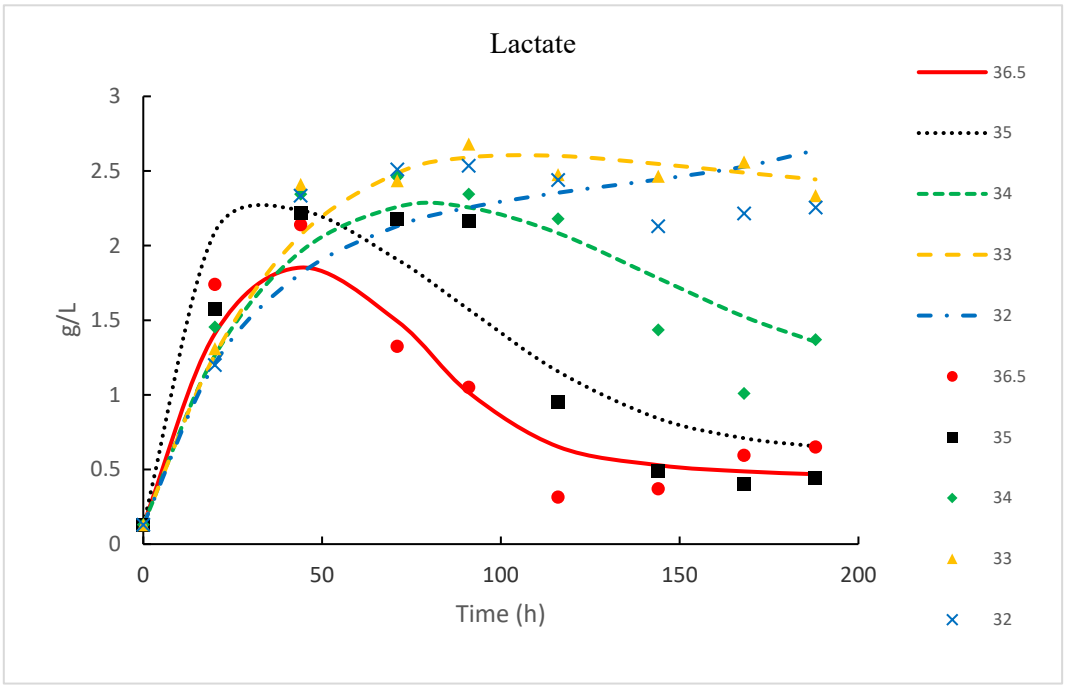
C


Glutamate
mM
Time (h)
36.5
35
34
33
32

D


Glucose
g/L
Time (h)
36.5
35
34
33
32

E



F

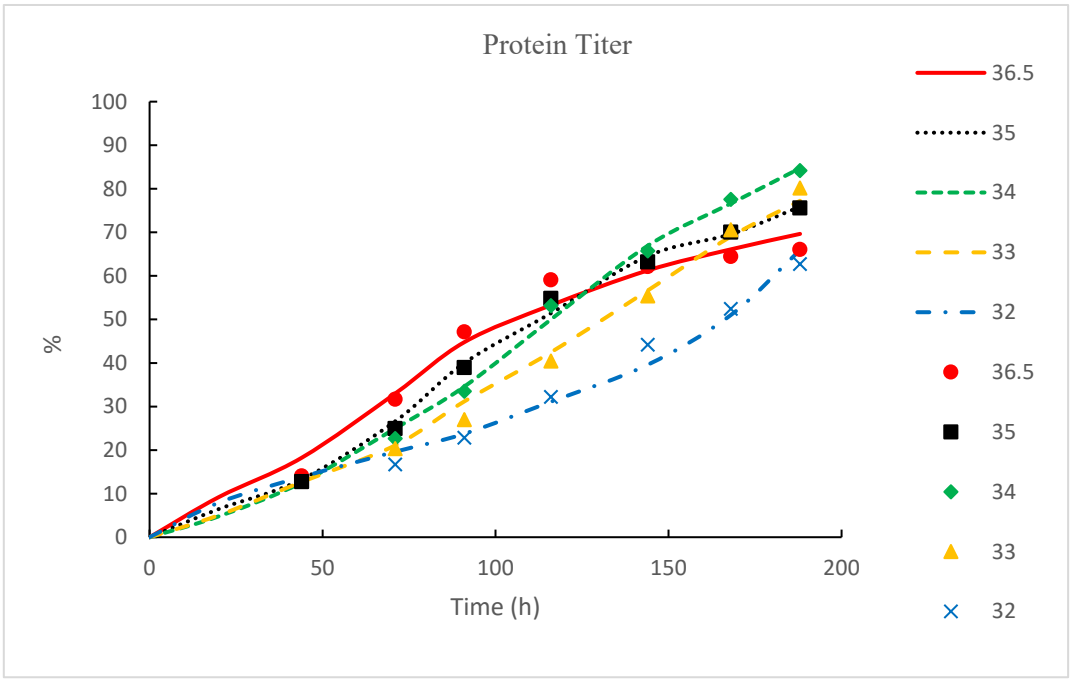


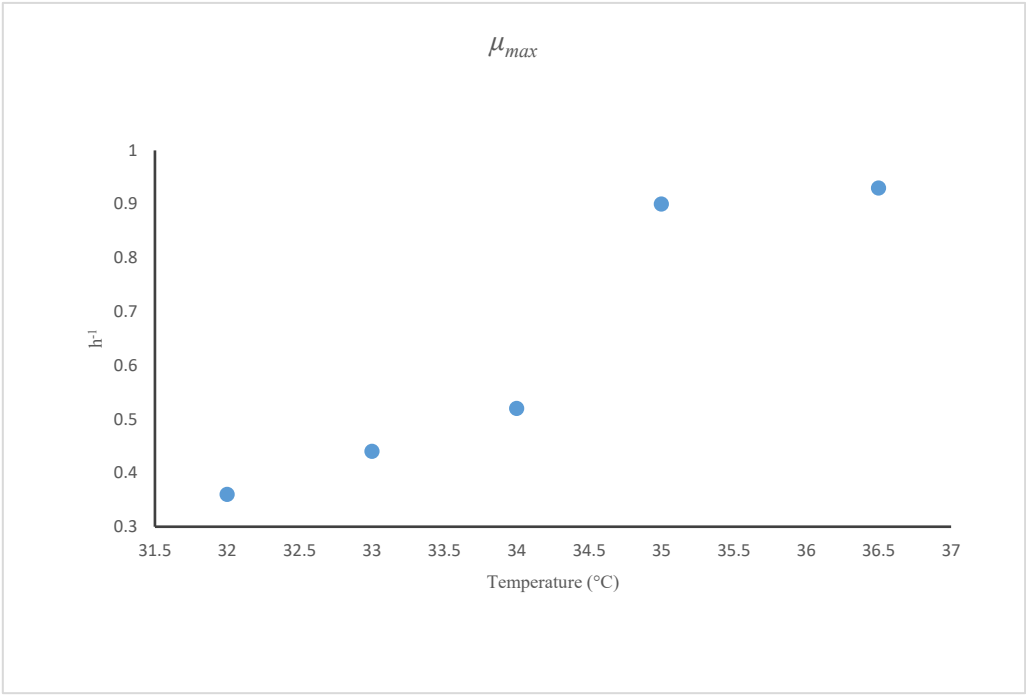
Figure 3-1. CHO-A profiles of VCD (**A**) and metabolites (**B**: glucose, **C**: glutamine, **D**: glutamate, **E**: lactate, **F**: normalized therapeutic protein titer. The points represent experimental data and the lines represent the simulation results. Calculated kinetic parameters are listed in Table 3-1.

Table 3-1. Key kinetic parameters of CHO-A

Temperature	°C	32	33	34	35	36.5	Ref.[96, 209, 227, 228]
Cell Growth	μ_{max} (h^{-1})	0.36	0.44	0.52	0.90	0.93	0.26 – 1.2
	k_d (day^{-1})	0.05	0.14	0.23	0.26	0.49	
Protein	KP $(g \cdot day^{-1} \cdot 10^{-9} cells^{-1})$	0.48	0.46	0.44	0.40	0.37	0.3 – 0.64
Glutamine	a_{Gln} $(mmol \cdot 10^{-10} cells^{-1} \cdot h^{-1})$	-3.6	-4.2	-4.9	-4.1	-5.7	
	YF_{Gln} $(10^6 cells \cdot mmol^{-1})$	37	52	86	104	183	
Glutamate	a_{Glu} $(mmol \cdot 10^{-11} \cdot cells^{-1} \cdot h^{-1})$	3.9	3.6	3.8	10	7.3	
	YF_{Glu} $(10^6 cells \cdot mmol^{-1})$	24	28	68	146	139	
Glucose	a_{Glc} $(g \cdot 10^{-13} cells^{-1} \cdot h\text{-}1)$	5.2	6.5	7.6	13	16	
	YF_{Glc} $(10^9 cells \cdot g^{-1})$	3.0	2.7	2.1	1.7	1.6	2.0 – 3.4
Lactate	a_{Lac} $(g \cdot 10^{-12} cells^{-1} \cdot h^{-1})$	10	8.1	5.5	4.6	4.1	
	b_{Lac} $(g \cdot 10^{-9} cells^{-1})$	0.83	1.2	1.6	2.3	5.6	
	k_{Lac} $(g \cdot 10^{-11} cells^{-1} \cdot h^{-1})$	0.87	1.1	1.1	1.8	2.6	
Ammonia	a_{Amm} $(mmol \cdot 10^{-9} \cdot cells^{-1} \cdot h^{-1})$	0.53	1.2	1.7	6.7	9.6	
	b_{Amm} $(mmol \cdot 10^{-10} \cdot cells^{-1})$	2.9	2.7	3.2	2.2	1.8	
	k_{Amm} $(mmol \cdot 10^{-9} cells^{-1} \cdot h^{-1})$	0.93	1.8	3.4	6.0	18.7	

Ref.: Values in references.

A



B

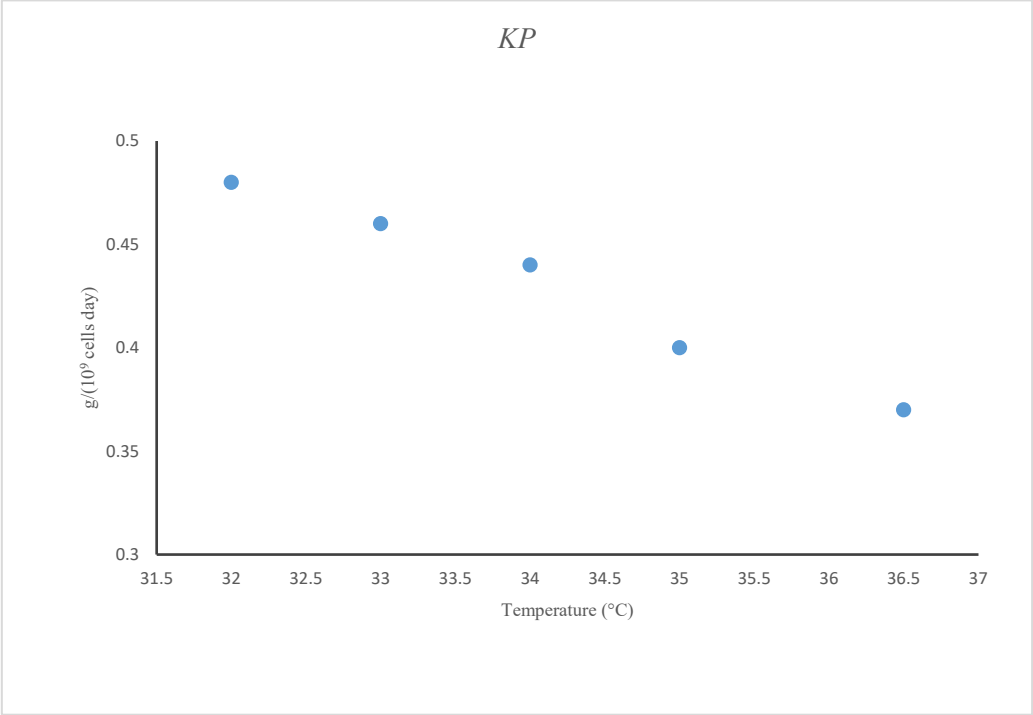


Fig. 3-2. Computed μ_{max} (**A**) and *KP* (**B**) values of CHO-A at different temperatures via kinetic models.

A trend of enhanced glutamine and glutamate utilization efficiency for cell growth was observed from the ascending YF_{Gln} and YF_{Glu} values with increasing temperature. Contrarily, descending YF_{Glc} and ascending a_{Glc} referred to lower glucose utilization efficiency with increasing temperature. This indicates that an appropriate hypothermia temperature shift with the studied temperature range may enhance the glucose utilization but may not favor the amino acids utilization efficiency for CHO-A.

Lactate metabolism was also sensitive to temperature. With increasing temperature, higher k_{Lac} and b_{Lac}, and lower a_{Lac} were obtained, implying that higher temperature accelerated lactate consumption but had less effect on lactate production. This explained the earlier lactate metabolism shift and lower lactate peak value at higher temperatures.

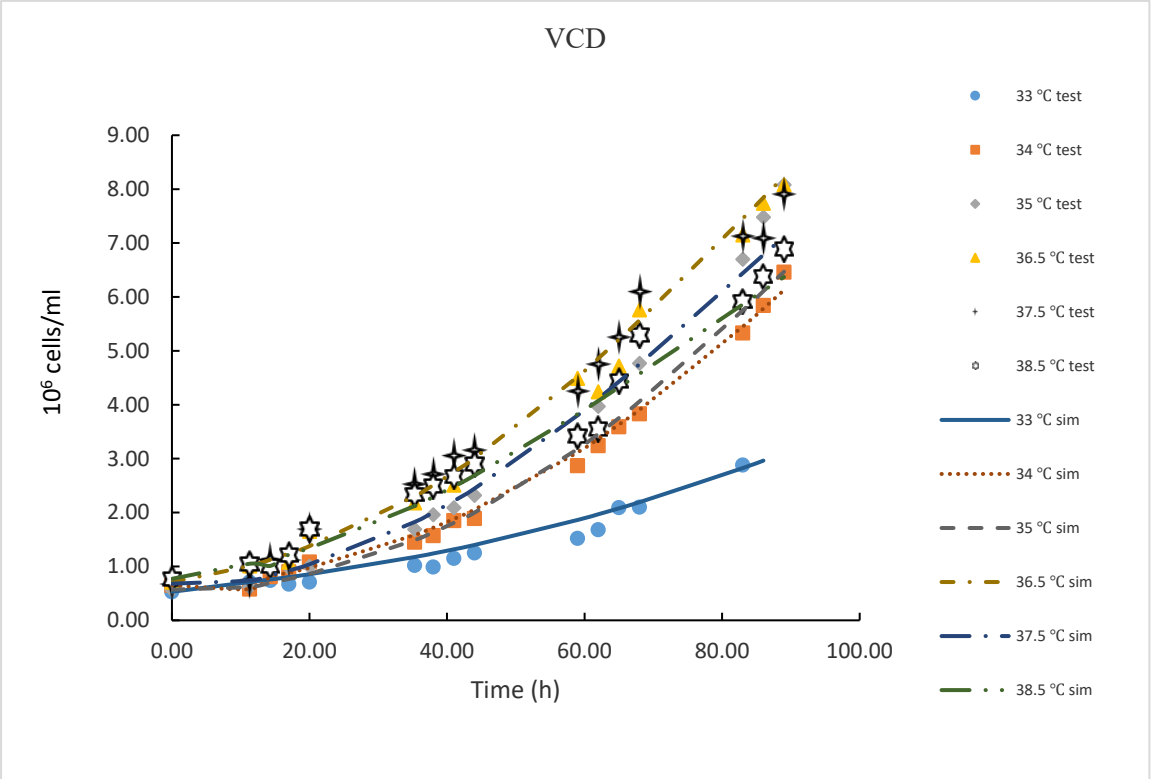
Though the highest therapeutic protein titer was observed at 34 ºC, the productivity (*KP*) was negatively correlated with temperature increase (Fig. 3-1), potentially due to the maintenance of active central metabolism, cell respiration and mitochondrial biogenesis [211]. Fig. 3-2 exhibited that μ_G and *KP* had opposite relevance with temperature. Because protein production was associated with both integral cell density and specific protein productivity, a TS strategy provides good balance of μ_G and *KP* were required to reach a high harvest therapeutic protein titer.

3.4.1.2.CHO-B

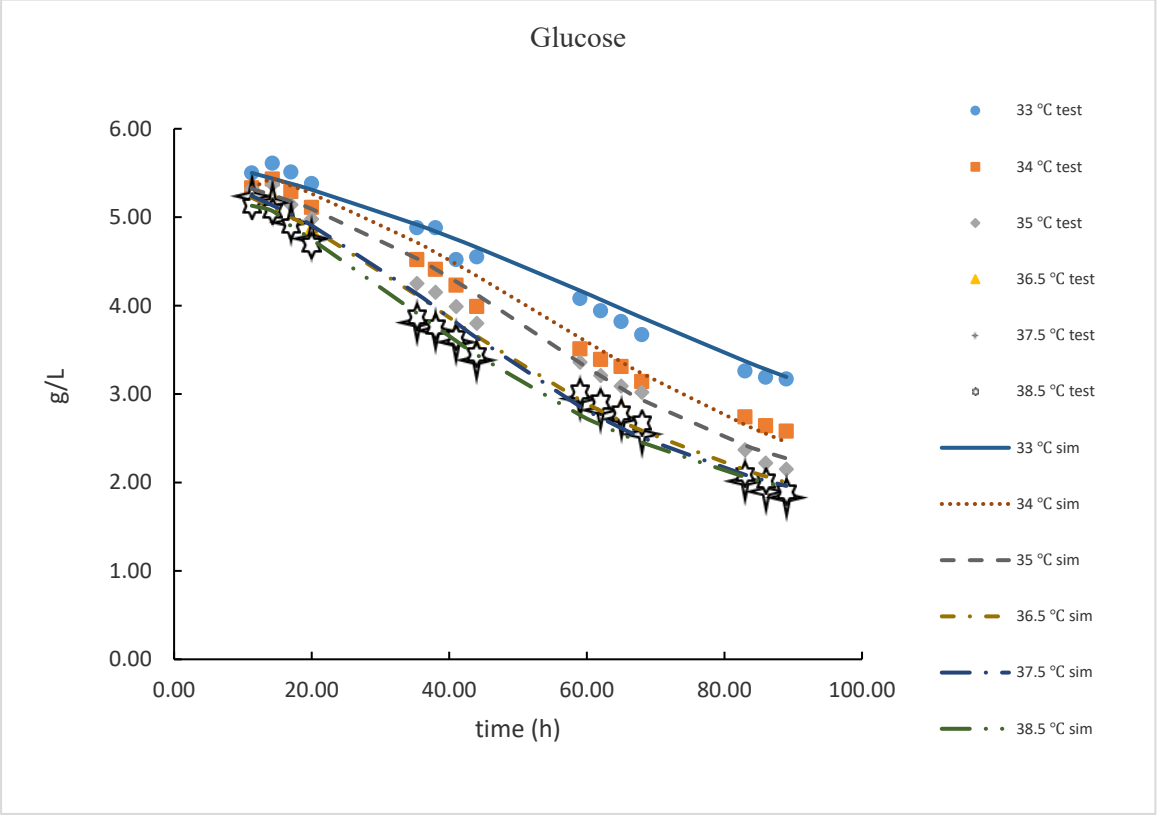
A 4-day batch cell culture behavior is shown in Fig. 3-3. Within the studied temperature range, μ_{max} doubled from 33 to 34 ºC and continued to increase slightly with ascending temperature from 34 to 38 ºC. Cell death rate (k_d) however increased

with ascending temperature. This indicates that a moderate temperature should be applied for fast VCD expansion. In contrast to CHO-A, CHO-B exhibited optimum temperatures at 35 and 36.5 ºC for high glucose (YF_{Glc}) and glutamate (YF_{Glu}) utilization. From 33 to 38.5 ºC, a_{Lac} and k_{Lac} were doubled while a_{Amm} and k_{Amm} exhibited little change (Table 3-2). This indicates that its lactate metabolism was more sensitive to temperature than ammonia. A TS strategy may have more effect on lactate metabolism adjustment than on ammonia.

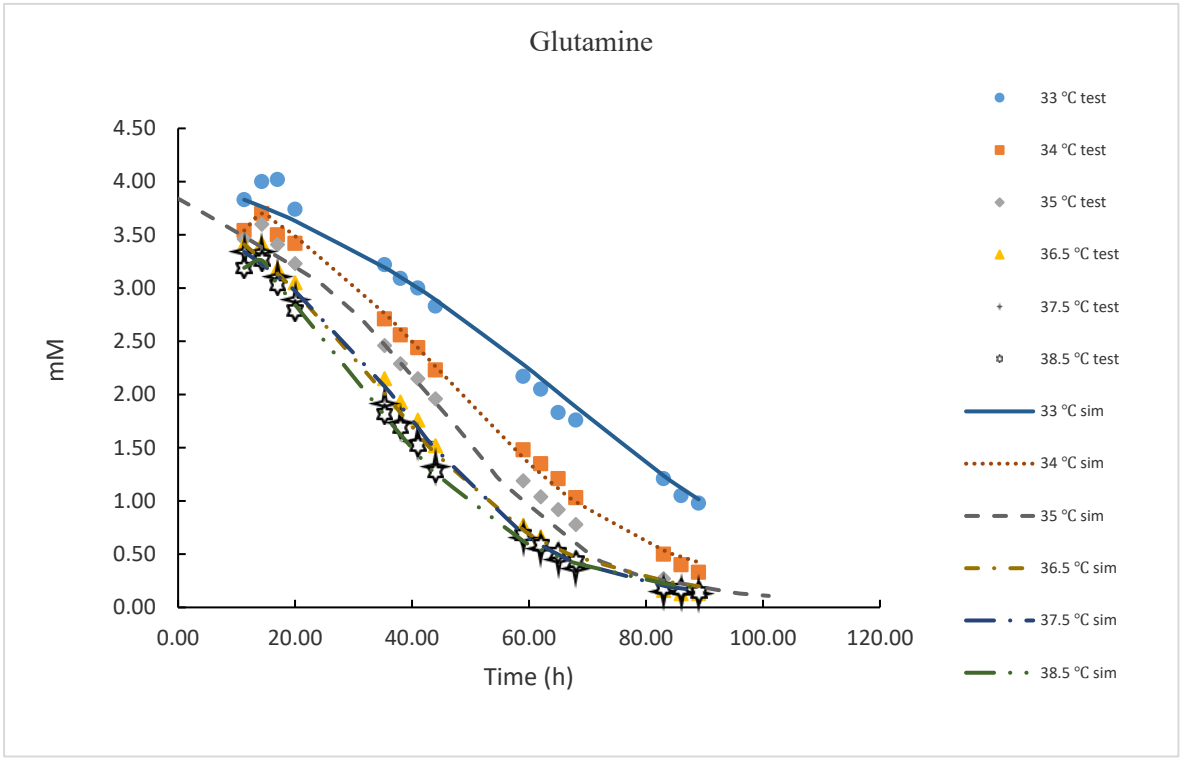
A


VCD
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
10^6 cells/ml
0.00
20.00
40.00
60.00
80.00
100.00
Time (h)
33 ℃ test
34 ℃ test
35 ℃ test
36.5 ℃ test
37.5 ℃ test
38.5 ℃ test
33 ℃ sim
34 ℃ sim
35 ℃ sim
36.5 ℃ sim
37.5 ℃ sim
38.5 ℃ sim

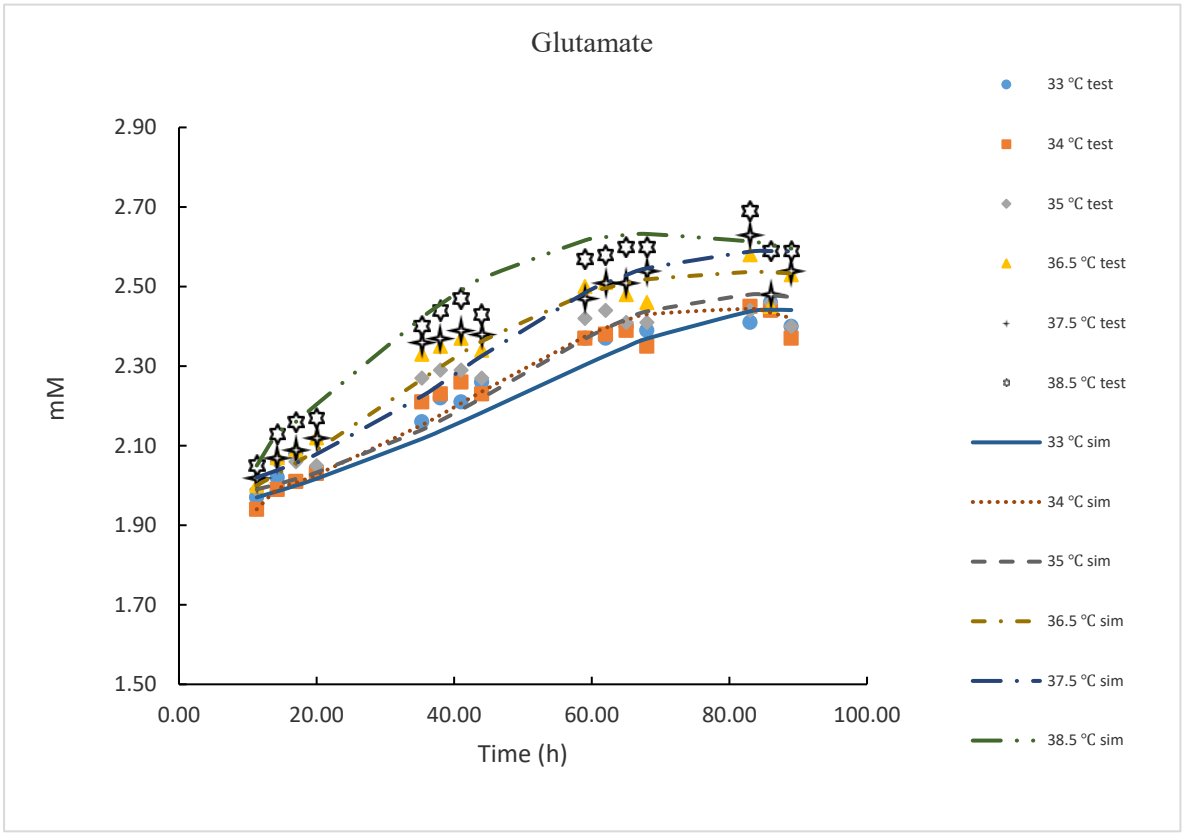
B


Glucose
6.00
5.00
4.00
3.00
2.00
1.00
0.00
g/L
0.00
20.00
40.00
60.00
80.00
100.00
time (h)
33 ℃ test
34 ℃ test
35 ℃ test
36.5 ℃ test
37.5 ℃ test
38.5 ℃ test
33 ℃ sim
34 ℃ sim
35 ℃ sim
36.5 ℃ sim
37.5 ℃ sim
38.5 ℃ sim

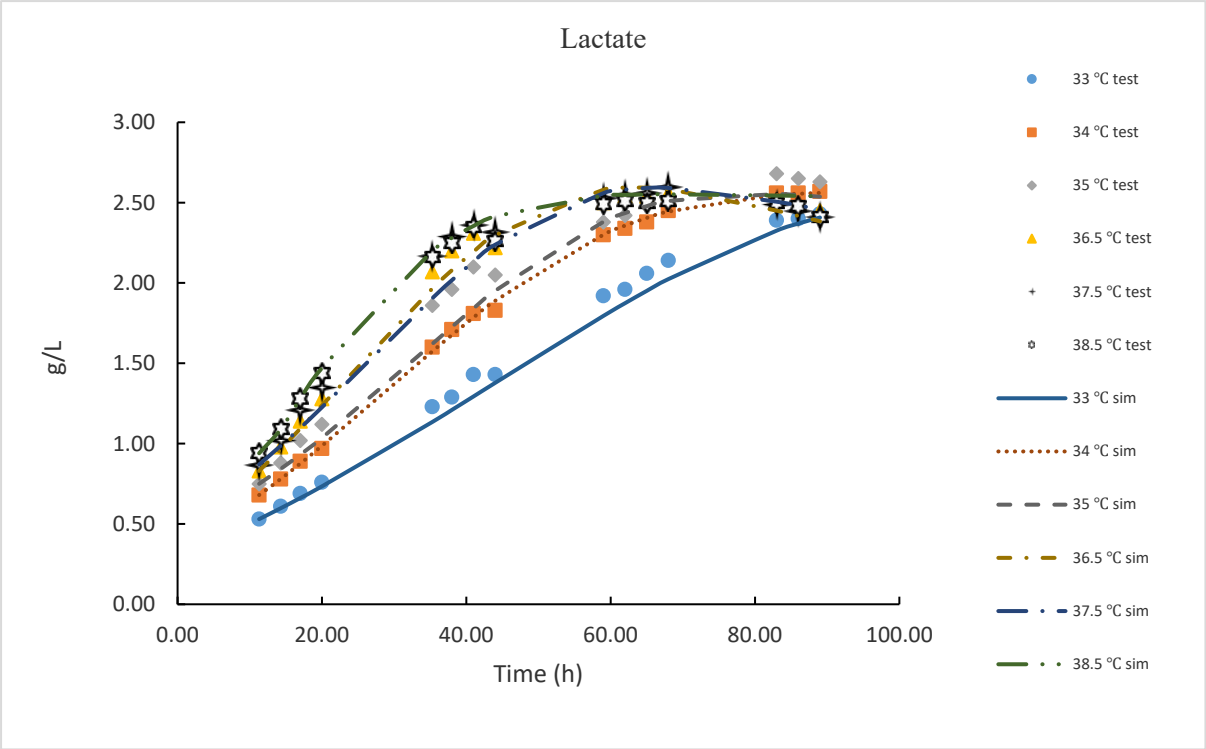
C


Glutamine
mM
Time (h)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.00
20.00
40.00
60.00
80.00
100.00
120.00
33 °C test
34 °C test
35 °C test
36.5 °C test
37.5 °C test
38.5 °C test
33 °C sim
34 °C sim
35 °C sim
36.5 °C sim
37.5 °C sim
38.5 °C sim

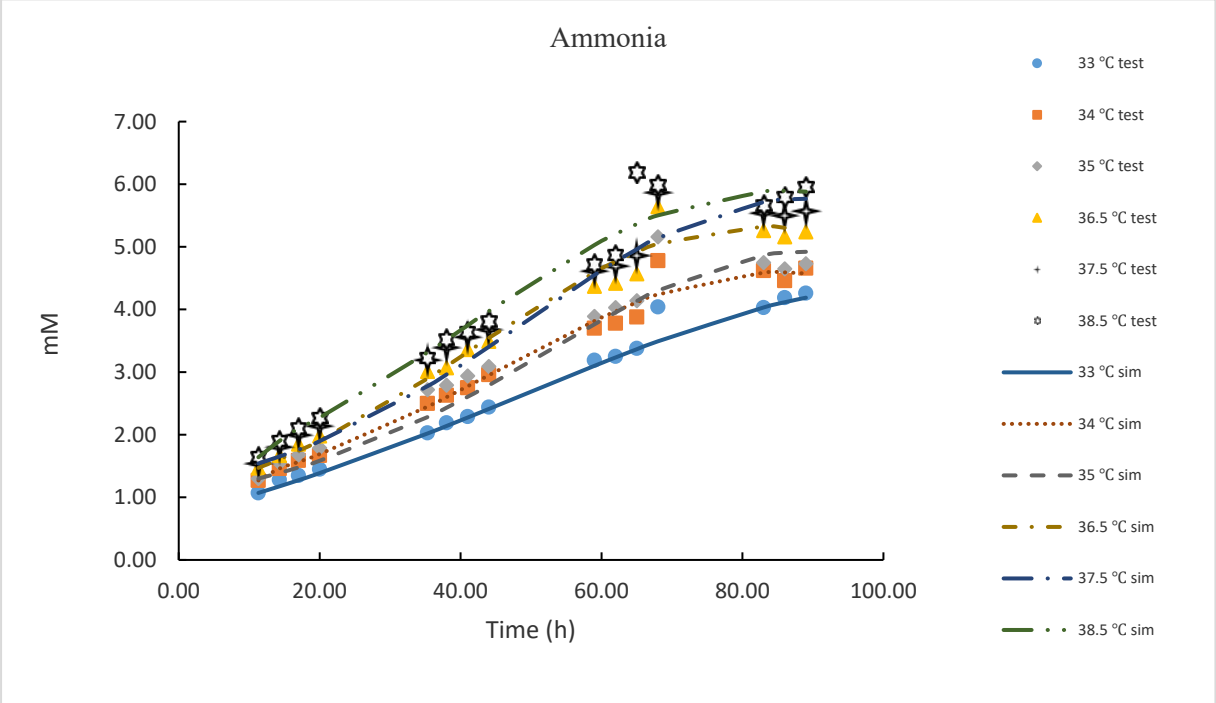
D


Glutamate
mM
Time (h)
2.90
2.70
2.50
2.30
2.10
1.90
1.70
1.50
0.00
20.00
40.00
60.00
80.00
100.00
33 °C test
34 °C test
35 °C test
36.5 °C test
37.5 °C test
38.5 °C test
33 °C sim
34 °C sim
35 °C sim
36.5 °C sim
37.5 °C sim
38.5 °C sim

E



F



Fig, 3-3. CHO-B profiles of VCD (**A**) and metabolites (**B**: glucose, **C**: glutamine, **D**: glutamate, **E**: lactate, **F**: ammonia. The points represent experimental data and the lines represent the simulation results. Calculated kinetic parameters are listed in Table 3-2.

Table 3-2. Key kinetic parameters of CHO-B

Temperature	°C	33	34	35	36.5	37.5	38.5
Cell Growth	μ_{max} (h^{-1})	0.059	0.116	0.121	0.126	0.128	0.120
	k_d (day^{-1})	0.048	0.065	0.084	0.13	0.13	0.14
Glutamate	YF_{Glu} ($10^6 cells \cdot mmol^{-1}$)	15	29	56	55	25	14
Glucose	YF_{Glc} ($10^9 cells \cdot g^{-1}$)	0.95	1.5	1.9	1.9	1.7	1.4
Lactate	a_{Lac} ($g \cdot 10^{-11} cells^{-1} \cdot h^{-1}$	4.0	5.0	5.0	7.7	8.0	8.1
	b_{Lac} ($g \cdot 10^{-10} cells^{-1}$)	2.0	1.6	1.4	1.4	1.1	0.76
	k_{Lac} ($g \cdot 10^{-11} cells^{-1} \cdot h^{-1}$)	2.2	2.8	2.9	4.3	4.4	4.4
Ammonia	a_{Amm} ($mmol \cdot 10^{-8} \cdot cells^{-1} \cdot h^{-1}$)	3	3	3	3	4	5
	b_{Amm} ($mmol \cdot 10^{-7} \cdot cells^{-1}$)	9.1	9.5	8.7	8.4	9.4	6.8
	k_{Amm} ($mmol \cdot 10^{-8} cells^{-1} \cdot h^{-1}$)	7.9	8.5	8.5	8.5	8.5	8.5

3.4.2. Temperature shift strategy prediction

3.4.2.1.Titer prediction for CHO-A

Using kinetic parameters determined in the previous section, the models (3.1) to (3.8) are used to compute the VCD and therapeutic protein profiles of CHO-A with a normal seeding density of 0.6×10^6 cells/ml at different TS strategies. While a continuous temperature shift condition may reports the highest titer, a current industry applicable TS strategy for manufacturing is one- or two-step shift. Table 3-3 listed the predicted titer on harvest day (day 14) based on 31 different TS strategies via kinetic models (3.1) - (3.8). These designed TS strategies include the temperature shift occurs on different days, via different steps, and to different temperatures, which should represent the typical TS strategies that could be applied for bioprocess development. 6

TS strategies were validated by experiments, which showed less than 3 % titer difference between the predicted and validated values. From the table, a day 10 shift from 36.5°C to 33°C reported the highest titer, which was set as 100 % normalized titer in this study.

Table 3-3. Predicted therapeutic protein titer via kinetic modeling

TS strategy	Shift day(s) (day)		Shift temperature (°C) [a]		Normalized predicted titer (%) [b]	Normalized validated titer (%) [c]	High titer candidates [d]
1	NA [e]				89.14		
2	6		32		82.07		
3			33		87.89	90.24	
4			34		92.29	92.32	
5			35		92.41		
6	8		32		87.34		
7			33		95.14	96.82	
8			34		97.77	97.83	*
9			35		97.98		*
10	10		32		92.11		
11			33		100.00		*
12			34		98.53		*
13			35		93.82		
14 [f]	6	8	35	32	87.18		
15				33	94.89		
16				34	97.43		*
17			34	32	87.27		
18				33	94.46	94.19	
19			33	32	83.27		
20	6	10	35	32	87.67		
21				33	95.17		
22				34	97.64		*
23			34	32	92.75		
24				33	94.80		
25			33	32	86.97		
26	8	10	35	32	88.13		
27				33	95.41		
28				34	97.86		*

29			34	32	98.10		*
30				33	95.14	95.11	
31			33	32	90.64		

[a] The temperature was kept at 36.5 °C before the temperature shift.

[b] Predicted titer on harvest day (day 14).

[c] Average titer measured in validation experiment, the experiment was operated in duplication.

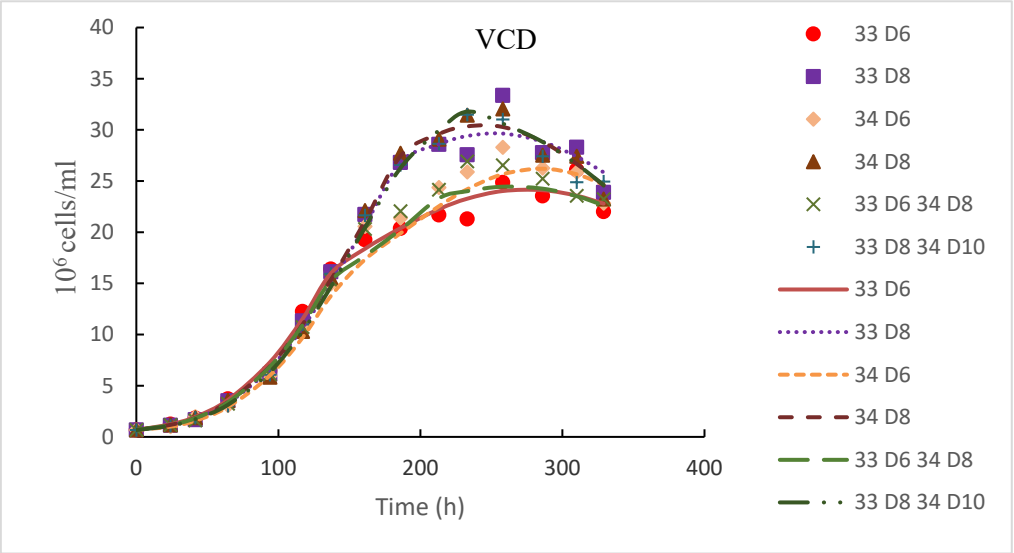
[d] The TS strategy candidates for high titer was marked with *. The qualified TS strategies was shortlisted based on its normalized predicted titer ≥ 97 %

[e] NA means the temperature was set to be constant at 36.5 °C without TS.

[f] The temperature shift occurred twice, first on day 6 to 35°C, and then on day 8 to 32°C. The same 'twice temperature shift' strategies were applied from No. 14 to No. 31 except the shifting dates and temperatures vary.

Considering the 3 % modeling deviation, the top productivity TS strategies can be screened based on the normalized predicted titer ≥ 97 % in Table 3. Thus, 8 TS strategies were shortlisted as candidates for high titer, while most of them includes a temperature shift to 33 ºC or 34 ºC. This means 33 ºC and 34 ºC ensured a good balance of cell growth (μ_G) and specific protein productivity (Q_{TP}), which agrees with the trends in Fig. 3-2. Instead of testing all the 31 TS strategies, this modeling method allows to narrow the candidate high productivity TS strategies to 8. It potentially saves 74 % of lab work and cost for bioprocess development.

A



B

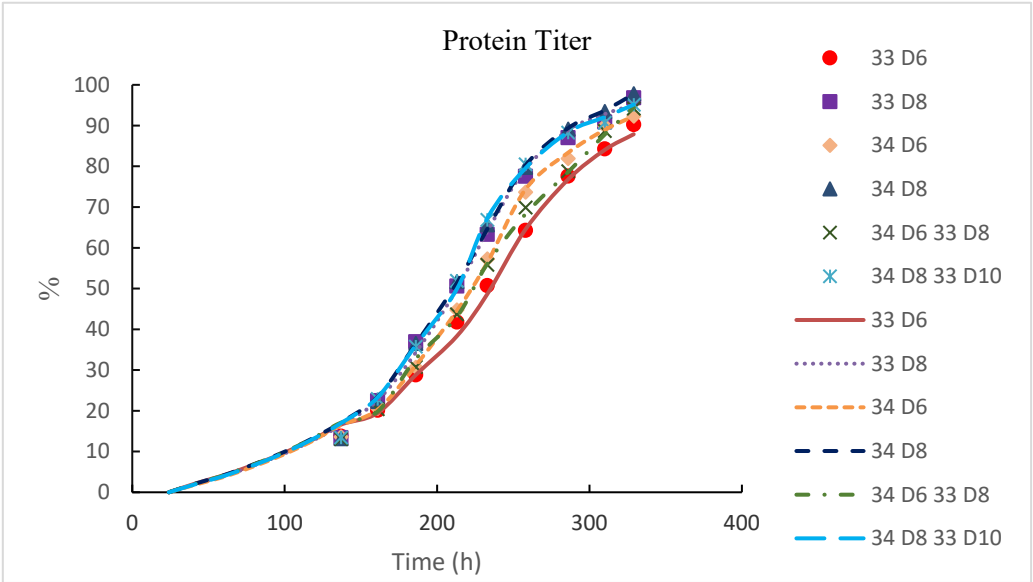


Fig. 3-4. VCD (**A**) and normalized protein (**B**) profiles and model validation of CHO-A computed at different TS strategies with an initial VCD of 0.6 × 10^6 cells/mL and temperature shifted from 36.5 °C to 33 or 34 °C on different days and steps. The lines denote to the computing results and the points represent the experimental data for model validation.

Fig. 3-4 showed that not only final titer but also the VCD and protein profiles were able to be well predicted, which was examined by the experiment validation. It indicates

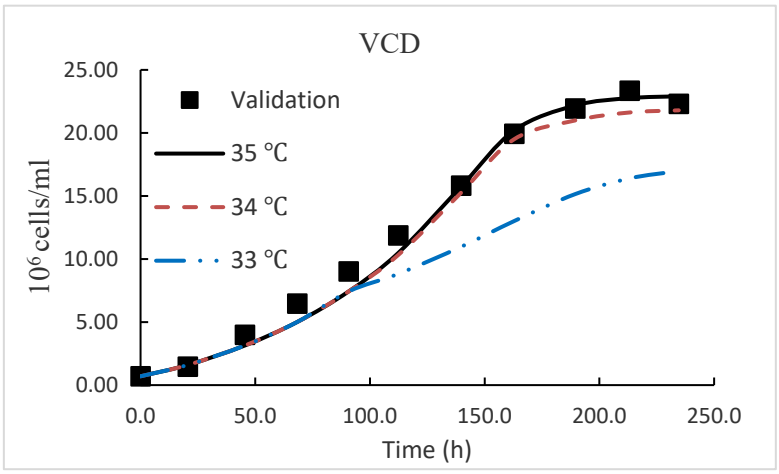
that the shifting time of day 6 or day 8 had more impact than temperature choice of 33 ºC or 34 ºC on VCD profile. A later shift supported a higher peak VCD. Meanwhile, both temperature and shift time were important for the protein titer. A later shift and higher shift temperature favored higher titer. However, a multi-step TS strategy that involved shifting to 34 ºC first and then to 33 ºC after two days, was also capable of reaching a similar level of protein titer as a single-step temperature shift strategy to 34 ºC, which was a comprehensive outcome of cell growth, death and protein expression.

Since temperature may have an impact on therapeutic protein quality, the listed multi-step TS strategies in Table 3-3 and Fig. 3-4 provide a choice to reach a similar protein titer at various temperatures, which may be employed to optimize the protein quality. Kinetic modeling provides an efficient means to predict the VCD and protein profiles for screening an optimal TS strategy, instead of testing all the possible TS strategies by experiments.

3.4.2.2.VCD prediction for CHO-B

For some CHO cell lines, it may hold the truth that that the *KP* are relatively inert with temperature. Thus, protein titer may heavily rely on VCD. Under this assumption, even shorter cell culture can be sufficient to contain the cell growth kinetic information, and further be applied as to predict the VCD profiles for rapid TS strategy screening. In this study, 4-day batch experiments of model cell line CHO-B at consistent temperatures were applied to predict the cell growth performance up to 10 days at various TS strategies, which targeted at significant reduce of lab operation.

A



B

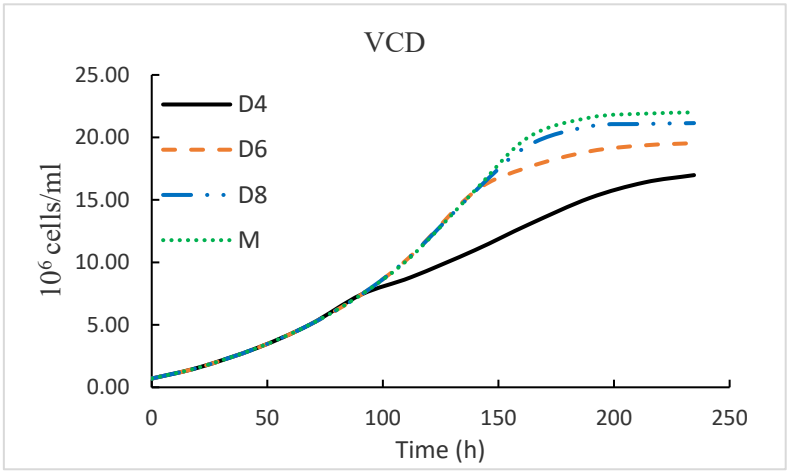


Fig. 3-5. VCD profiles prediction and validation of CHO-B at different TS strategies with an initial VCD of 0.6 × 10^6 cells/mL: TS from 36.5 °C to 35, 34 and 33 °C respectively on day 4 (**A**); TS from 36.5 °C to 33 °C on day 4, day 6 and day 8, respectively (**B**). **M** represents a multiple temperature shifting strategy: TS from 36.5 °C to 35 °C on day 4, then to 34 °C on day 6, and finally to 33°C on day 8. The lines denote the computational prediction results and the points represent the experimental data for validation.

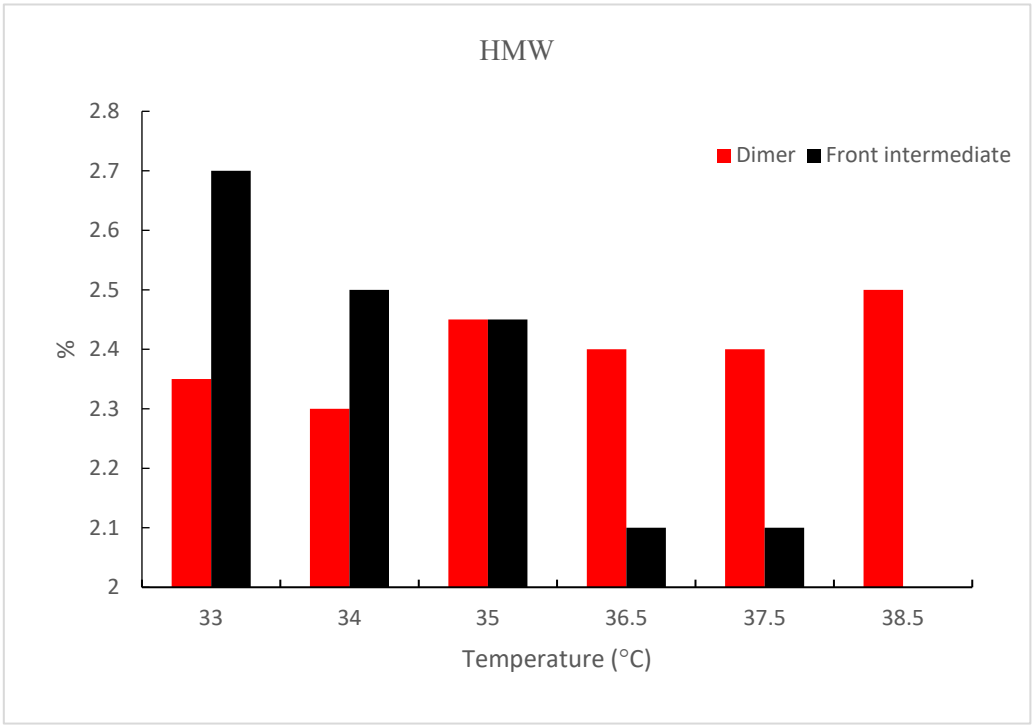
Fig. 3-5 showed the VCD profiles of CHO-B computed via kinetic models at

different TS strategies in a fed-batch mode, which validated by the experimental results. It indicated that higher shifting temperature and late TS time were beneficial for biomass accumulation in this case. Specifically, a multi-step TS starting from day 4 was also capable of achieving a similar VCD as TS at day 8, which indicated that less disturbance would apply stress on cell growth by gradual TS [210].

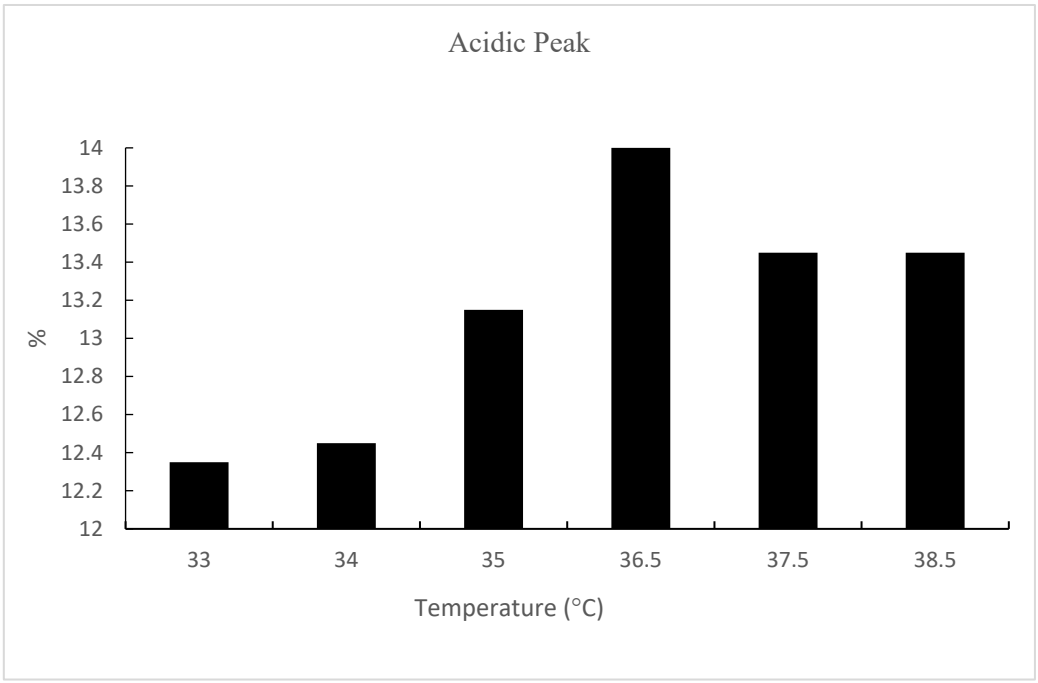
3.4.3. Therapeutic protein quality attributes

Temperature affects not only biomass accumulation and therapeutic protein productivity, but also protein quality attributes [210, 211], including high molecular weight (HMW) [229], charge variants [230, 231], and N-linked glycosylation [72, 232]. Instead of detecting after a full period cell culture, a short duration of 2-day batch run of CHO-B with a high seeding density of 10×10^6 cells/ml was used to characterize the trends of temperature effect on the protein quality attributes (Fig. 3-6). After 2-day cell culturing, variation of quality attributes could be observed. A hypothermal operation favored a high percentage of front intermediate, while an appropriate megathermal operation reported a high Man5 percentage. Temperature below 36.5 ºC generally reported lower acidic peak and higher basic peak than that above 36.5 ºC. Meanwhile a maximum acidic peak and minimum basic peak was observed at 36.5 ºC.

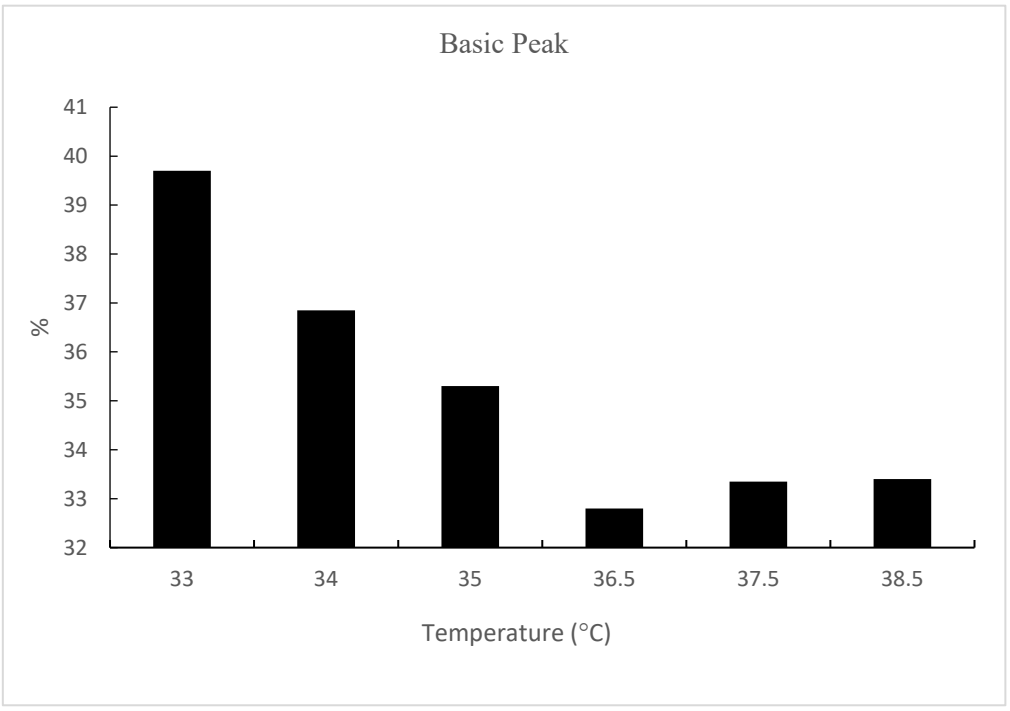
A


HMW
Dimer
Front intermediate
%
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2
33
34
35
36.5
37.5
38.5
Temperature (°C)

B


Acidic Peak
%
14
13.8
13.6
13.4
13.2
13
12.8
12.6
12.4
12.2
12
33
34
35
36.5
37.5
38.5
Temperature (°C)

C



D

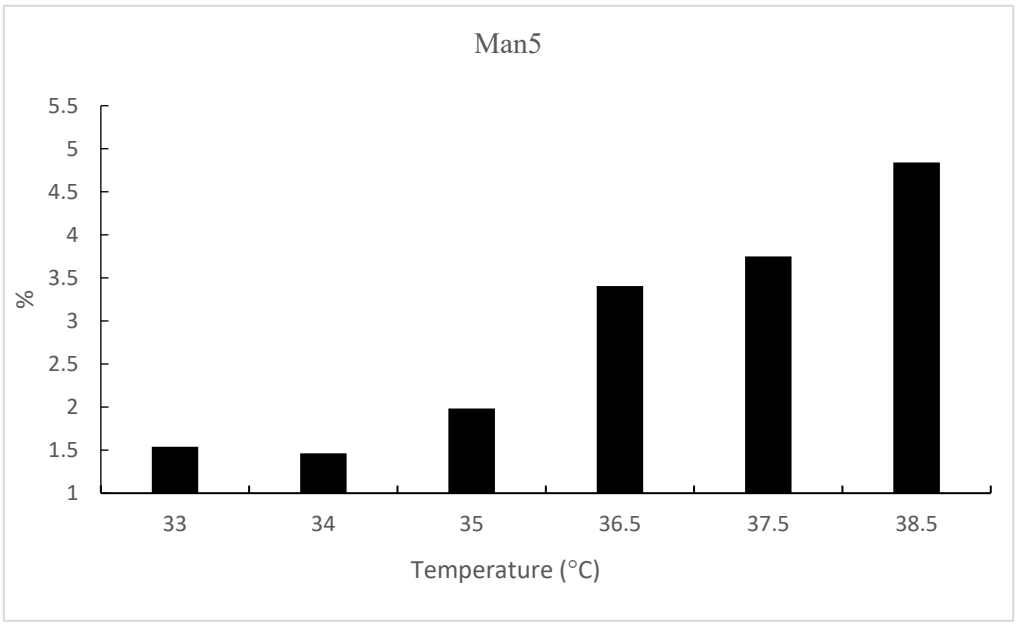


Fig. 3-6. Impact of cell culture temperature on protein quality attributes including HMW (**A**), charge variant (**B & C**), and Man5 (**D**). CHO cell line B cultured in a 125-mL batch shake flask runs with a high initial VCD of 10×10^6 cells/mL for 2 days.

3.5.Conclusion

The temperature effect on CHO cell bioprocess from 32 to 36.5 °C and from 33 to 38.5 °C for two CHO cell lines were systematically studied via kinetic modeling. These two CHO cell lines exhibited different responses to temperature change on the aspects of cell growth, therapeutic protein productivity, and nutrients and metabolites metabolism.

Kinetic modeling was applied for rapid TS strategy screening and prediction. A series of 8-day CHO cell culture with high seeding density of 10×10^6 cells/ml at different constant temperatures were applied to predict 14-day CHO cell culture processes with normal seeding density of 0.6×10^6 cells/ml at different TS strategies. Meanwhile, a series of 4-day batch CHO cell culture which contained sufficient exponential phase kinetic information, was applied to predict the bioprocesses to an extended period of 10 days in a fed-batch mode. Therapeutic protein quality attributes in short duration experiments exhibited the trends of those in the 14-day cell culture, which could also be potentially applied for indicating the full-duration cell culture.

Owing to the short time needed for each temperature shift, sudden temperature shift or step changes were applied in the kinetic modeling study. However, it may take hours to reach the down shifting temperature in the manufacturing scale bioreactor (2,000 – 25,000 L). Step change in temperature may not be applicable. This study was performed with two model CHO cell lines, the general methodology developed here is applicable to other therapeutic protein production by mammalian cells with reduced process development time and financial investment.For a better understanding of cell culture,

an insight view of the intracellular enzymes is beneficial. The next chapter will provide a mechanistic modeling method to elucidate oligomeric enzyme kinetic behaviors.

Chapter 4

A Mechanistic Kinetic Description of Lactate Dehydrogenase Elucidating Cancer Diagnosis and Inhibitor Evaluation

4.1. Abstract

As a key enzyme for glycolysis, lactate dehydrogenase (LDH) remains as a topic of great interest in cancer study. Though a number of kinetic models have been applied to describe the dynamic behavior of LDH, few can reflect its actual mechanism, making it difficult to explain the observed substrate and competitor inhibitions at a wide substrate concentration range. A novel mechanistic kinetic model is developed based on the enzymatic processes and the interactive properties of LDH. Better kinetic simulation as well as new enzyme interactivity information and kinetic properties extracted from published articles via the novel model was presented. Case studies were presented to a comprehensive understanding of the effect of temperature, substrate, and inhibitor on LDH kinetic activities for promising application in cancer diagnosis, inhibitor evaluation and adequate drug dosage prediction.

Keywords: cancer diagnosis; inhibitor evaluation; kinetic model; lactate dehydrogenase; oligomeric enzyme

4.2.Introduction

Lactate dehydrogenase (LDH) is an essential enzyme in nearly all living cells [233, 234], which catalyzes the mutual transformation between pyruvate and lactate, associated with nicotinamide adenine dinucleotide compounds (NADH and NAD^+) interconversion [235] (Fig. 4-1). Because cancer cells heavily rely on aerobic glycolysis to support their growth, LDH comes to be an emerging anticancer target for cancer diagnosis and treatment [236]. Besides the enzyme level and catalytic mechanism, its kinetic properties have also attracted wide interests [91, 237]. From 1997 to 2016, approximately 1625 publications are found as LDH kinetic related, based on our query and manual filtration on a peer-reviewed literature database named as Scopus®.

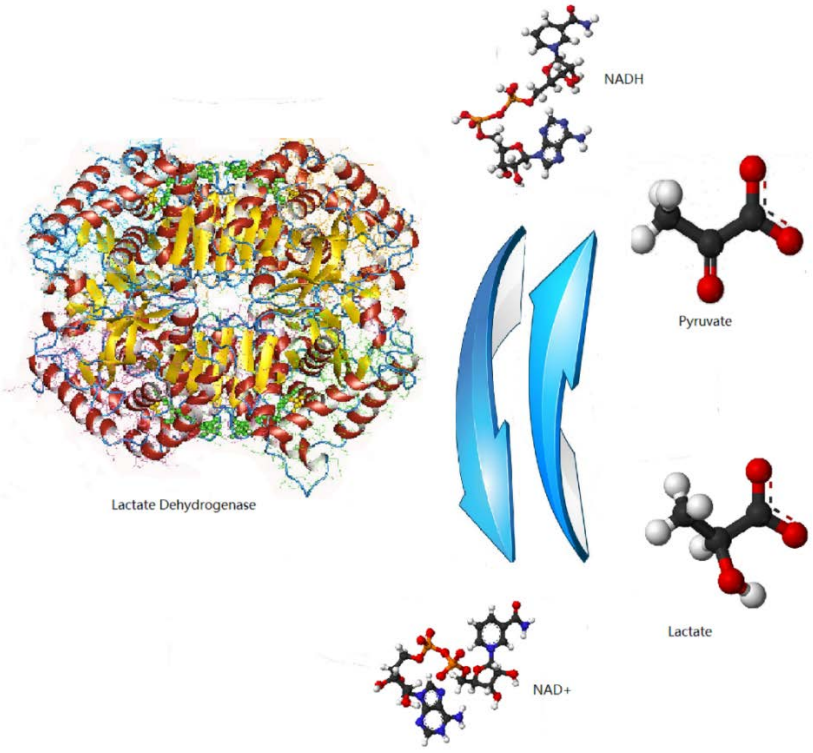


Fig. 4-1. Scheme of mutual transformation of pyruvate and lactate catalyzed by lactate dehydrogenase.

To inhibit the glycolysis within cancer cells, hundreds of small molecules are under study to reduce LDH activity. One type of inhibitor candidates are molecules that have similar chemical structures as pyruvate and are able to competitively associate on the substrate domain. Oxamate derivatives (Fig. 4-2), for example, are one type of model inhibitors [238]. These molecules are able to seize the available substrate-binding sites and further inhibit substrate binding and reactivity. Meanwhile, tumor cells respond differently from normal cells to temperature changes [239]. Therefore, a universal method to quantitatively describe the kinetic properties of LDH and evaluate the effect of inhibitors and temperature can be beneficial for cancer study.

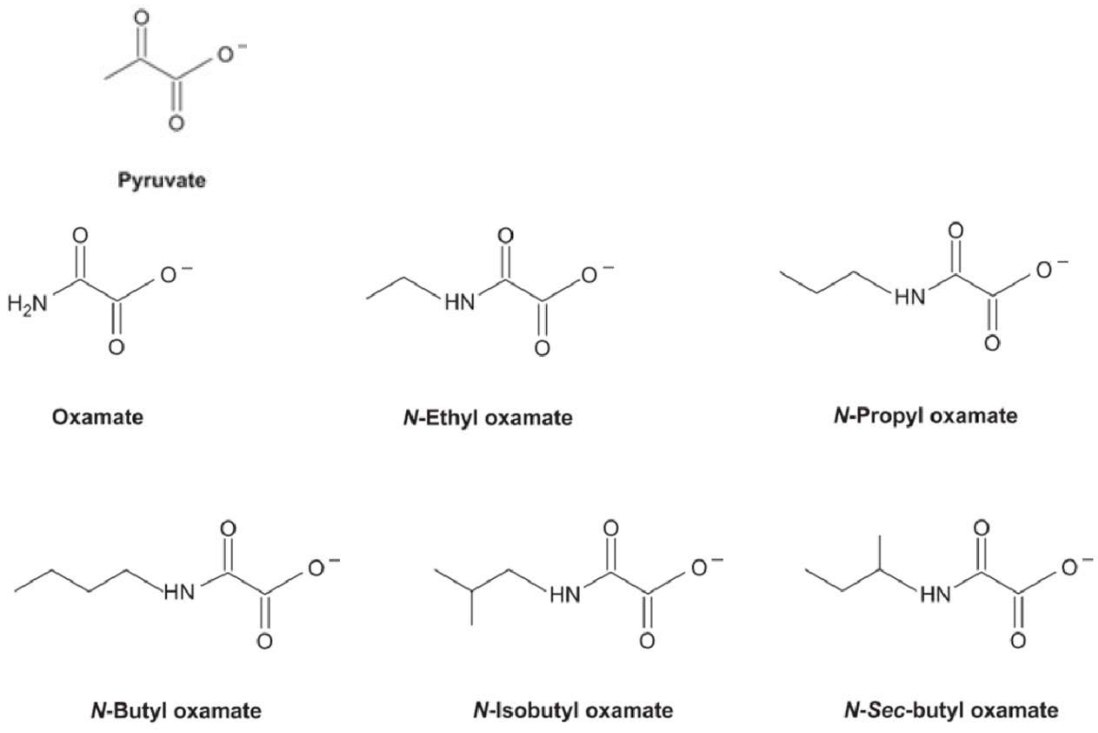


Fig. 4-2. Structures of pyruvate and oxamate derived inhibitors [240].

As a tetramer existing in most physiological environments [241, 242], one LDH molecule has four subdomains, and thus, is able to bind up to four substrate molecules and four coenzyme molecules. The binding of one or more substrate and coenzyme molecules regulates the oligomeric enzyme protein folding, leading to dynamic enzyme activity change. Thus, LDH is an interactive enzyme known to possess allosteric properties [89, 243].

Several kinetic models have been applied to describe LDH performance. However, few are able to elucidate the enzymatic process mechanistically. Known models include Michaelis-Menten (M-M) Equation, Hill Equation [94, 97, 103, 244], Binding Model of Alberty [105, 245], Monod-Wyman-Changeux (MWC) Model [89, 90], and empirical models derived from software of JMP [105]. The deficiency of these models is mainly the failure in taking a comprehensive consideration of the complex interactive processes and allosteric properties of the oligomeric enzyme.

The objective of this chapter is to develop a mechanistic kinetic model based on the interactive enzyme properties that is capable of describing temperature, substrate and inhibitor effect. It aims at better kinetic simulation of the enzymatic process, a comprehensive understanding of the enzyme behavior in complex conditions, as well as the promising applications in cancer diagnosis, inhibitor evaluation and adequate medication dosage.

4.3.Model Development

Due to the interactive nature, an oligomeric enzyme exhibits unique kinetic

properties at each successive binding. Thus, a mechanistic kinetic model needs to take all the information of enzyme interactive regulations caused by sequential coenzyme, substrate, and inhibitor binding into consideration. In order to better understand the enzyme kinetic properties and simplify the mathematic formula, this study focusses on the initial reaction rates. An isoenzyme consisted of identical subunits is used as the modeling example. Therefore, the binding and dissociation processes can be expressed as

$E_nS_{i-1} + S \longrightarrow E_nS_i, \qquad r_{+ni} = (n\text{-}i+1)\, k_{ni+}[E_nS_{i-1}][S]$ [4.1-a]

$E_nS_i \longrightarrow E_nS_{i-1} + S, \qquad r_{-ni} = ik_{ni-}[E_nS_i]$ [4.1-b]

Where, n is the total number of available binding sites of the oligomeric enzyme E_n, S denotes for the binding substrate, E_nS_i denotes for one enzyme molecule bound with i substrate molecules, k_{ni+} is the binding rate constant for substrate molecule on each of the $(n\text{-}i+1)$ vacant sites, and k_{ni-} is the dissociation of a substrate molecule from each of the i bound substrate molecules.

It is assumed that the binding is much faster than the reaction. Thus, thermodynamic equilibrium applies to substrate binding on all the sites, which is

$$[E_nS_i] = \frac{(n-i+1)k_{ni+}[E_nS_{i-1}][S]}{ik_{ni-}} = \binom{n}{i}\frac{[E_n][S]^i}{K_n\prod_{j=1}^{i-1}(\alpha_{nj}K_n)} \qquad [4.2]$$

where

$$\binom{n}{i} = \frac{n\cdot(n-1)\cdots(n-i+1)}{1\cdot 2\cdots i} \qquad [4.3\text{-a}]$$

$$K_n = \frac{k_{n1-}}{k_{n1+}} \qquad [4.3\text{-b}]$$

$$\alpha_{ni-1} = \frac{k_{ni-}}{k_{ni+}}\left(\frac{k_{n1-}}{k_{n1+}}\right)^{-1} \qquad [4.3\text{-c}]$$

Considering the mass balance of the enzyme leads to

$$E_n = [E_n] + \sum_{i=1}^{n}[E_n S_i] = [E_n] + \frac{[E_n][S]}{K_n}\sum_{i=1}^{n}\binom{n}{i}\frac{[S]^{i-1}}{\prod_{j=1}^{i-1}(\alpha_{nj}K_n)} \qquad [4.4]$$

Substituting equation [4.4] to [4.2] yields

$$[E_n S_i] = \frac{E_n\binom{n}{i}\frac{[S]^{i}}{\prod_{j=1}^{i-1}(\alpha_{nj}K_n)}}{K_n + [S]\sum_{k=1}^{n}\binom{n}{k}\frac{[S]^{k-1}}{\prod_{j=1}^{k-1}(\alpha_{nj}K_n)}} \qquad [4.5]$$

The overall catalytic reaction rate for an oligomeric enzyme with n-reactive binding sites, r_{Pn}, is then given by

$$r_{Pn} = k_n[E_n S] + k_n\sum_{i=2}^{n} i\beta_{ni-1}[E_n S_i] \qquad [4.6]$$

Where, β is the coefficient indicating the interactive effect on reactivity.

4.4. **Results**

4.4.1. LDH Saturated with coenzyme

LDH, as an interactive tetrameric enzyme, follows the general mechanistic kinetic principles expressed above. In studies conducted to examine the substrate (pyruvate or lactate) effect on LDH kinetic activities, an excess of coenzyme (NAD^+ or NADH) is generally added to saturate and fully activate the enzyme [246, 247]. Based on the reaction mechanism (Fig. 4-3) and equations [4.1] to [4.6], the reaction rate can be expressed as

$$r = 4\frac{kE[S]}{Kf}\left(1+\frac{3\beta_1[S]}{\alpha_1 K}+\frac{3\beta_2[S]^2}{\alpha_1\alpha_2 K^2}+\frac{\beta_3[S]^3}{\alpha_1\alpha_2\alpha_3 K^3}\right) \quad [4.7]$$

Where, E is the coenzyme saturated LDH isoenzyme, which is the same as E_4. f is defined as

$$f = 1+\frac{4[S]}{K}+\frac{6[S]^2}{\alpha_1 K^2}+\frac{4[S]^3}{\alpha_1\alpha_2 K^3}+\frac{[S]^4}{\alpha_1\alpha_2\alpha_3 K^4} \quad [4.8]$$

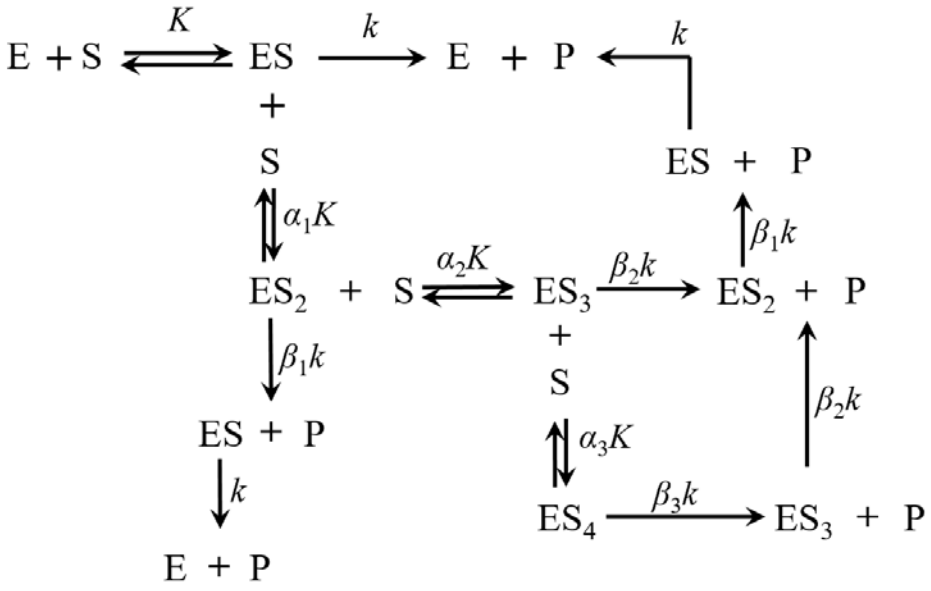


Fig. 4-3. Sequential substrate binding and reaction processes on LDH at the coenzyme saturation condition.

While it is conceivable to have the affinities vary extensively during the subsequent binding, there could be a pattern of affinity change. In this case, the cooperative effect is significantly simplified. If we let

$$\alpha_1 = \alpha_2 = \alpha_3 = \alpha \quad [4.9\text{-a}]$$

$$\beta_1 = \beta_2 = \beta_3 = \beta \quad [4.9\text{-b}]$$

The model now signifies that the successive binding approaches the same incremental

proportional affinity change.

Then, equation [4.7] can be simplified to

$$r = \frac{4kE[S]}{K} \frac{1 - \beta + \beta\left(1 + \frac{[S]}{\alpha K}\right)^3}{1 - \alpha + \alpha\left(1 + \frac{[S]}{\alpha K}\right)^4} \qquad [4.10]$$

Specifically, at a low substrate level, equation [10] can be further simplified to

$$r = \frac{kE[S]}{\frac{K}{4} + [S]} \qquad [4.11]$$

Equation [4.11] is the familiar M-M formula. Reaching this result after applying the aforementioned conditions explains how the M-M model can approximately simulate the kinetic process at low substrate concentrations. Equation [4.10] is in agreement with the conventional M-M model but is applicable outside of the limited condition.

Generally, $\alpha < 1$ is favorable for subsequent substrate binding; $\beta > 1$ indicates that subsequent binding accelerates the on-site reaction.

4.4.2. LDH competitive effector binding model

Inhibitors, such as oxamate derived molecules (Fig. 4-2), can seize the LDH substrate binding sites (Fig. 4-4) and reduce the enzyme activity. Therefore, at the coenzyme saturated condition, the process with competitive effectors can be expressed as

$$r = \sum_{i=1}^{4} \sum_{j=0}^{4-i} i\beta_{j,i-1} k[EI_j S_i] \qquad [4.12]$$

$$[EI_j] = \frac{(5-j)[EI_{j-1}][I]}{j\gamma_{j-1,0} K_I} \qquad (1 \le j \le 4) \qquad [4.13\text{-a}]$$

$$[ES_i] = \frac{(5-i)[ES_{i-1}][S]}{i\alpha_{0,i-1}K_S} \qquad (1 \le i \le 4) \qquad \text{[4.13-b]}$$

$$[EI_jS_i] = \frac{1}{2}\left\{\frac{(5-j-i)[EI_jS_{i-1}][S]}{i\alpha_{j,i-1}K_S} + \frac{(5-j-i)[EI_{j-1}S_i][I]}{j\gamma_{j-1,i}K_I}\right\} \qquad (1 \le i, j, i+j \le 4)$$

[13-c]

$$E = \sum_{i=0}^{4}\sum_{j=0}^{4-i}[EI_jS_i] \qquad \text{[4.14]}$$

Where, S and I are the substrate and inhibitor, respectively; k is the reaction rate constant of the first binding substrate without inhibitor; K is the saturation coefficient of the first binding of substrate (K_S) or inhibitor (K_I); α, β and γ are the interactive coefficients. The subscripts i and j associated with I and S indicate the number of substrate and inhibitor molecules bound to the enzyme complex, respectively; α and γ indicate the interactive binding effect on subsequent substrate and inhibitor, respectively; β indicates the interactive effect on reactivity.

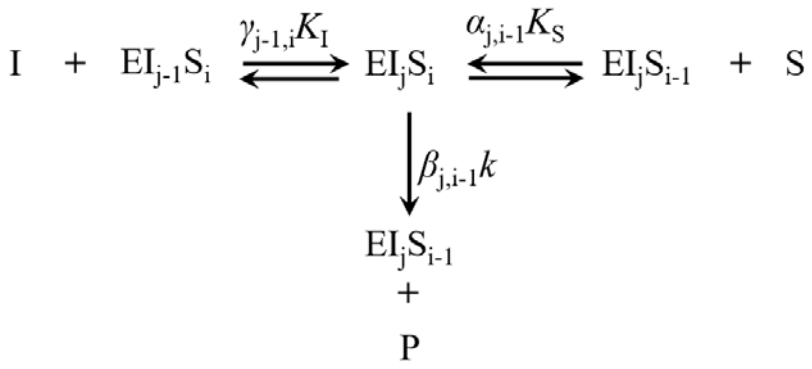


Fig. 4-4. Typical substrate and inhibitor binding and reaction processes. ($1 \le i, j, i+j \le 4$). S, P and I are the substrate, product, and competitive effector, respectively; α, β and γ represent the kinetic constants change due to interactive effects.

Following a similar assumption as equation [4.9-a] and [4.9-b], we let

$$\alpha_{m,n} = \alpha \qquad (1 \le m+n \le 3) \qquad \text{[4.15-a]}$$

$$\beta_{0,m} = \beta_S \qquad (1 \le m \le 3) \qquad \text{[4.15-b]}$$

$$\beta_{n,m} = \beta_{IS} \qquad (1 \le n, n+m \le 3) \qquad \text{[4.15-c]}$$

$$\gamma_{n,m} = \gamma \qquad (1 \le m+n \le 3) \qquad \text{[4.15-d]}$$

Where, subscript $_S$ indicates no inhibitor bound on the enzyme molecule and the interactive effect is only caused by subsequent substrate binding; subscript $_{IS}$ indicates that the interactive effect is caused by both inhibitor and substrate molecules bound on the enzyme complex.

Substituting equations [4.13], [4.14] and [4.15] into [4.12] leads to

$$r = \frac{4kE[S]}{K_S F}\left\{1-\beta_S+\beta_S\left(1+\frac{[S]}{\alpha K_S}\right)^3+\frac{\beta_{IS}[I]}{\gamma K_I}\left(\frac{3}{2}+\frac{3\lambda}{2\alpha}+\frac{3[I]}{4\gamma K_I}\left(1+\frac{3\gamma}{\alpha}\right)\right.\right.$$

$$\left.\left.+\frac{[I]^2}{8(\gamma K_I)^2}\left(1+\frac{7\gamma}{\alpha}\right)+\frac{[S]}{\alpha K_S}\left(\frac{9}{2}+\frac{3\lambda}{2\alpha}+\frac{3[S]}{8\alpha K_S}\left(7+\frac{\gamma}{\alpha}\right)+\frac{3[I]}{2\lambda K_I}\left(1+\frac{\gamma}{\alpha}\right)\right)\right)\right\} \qquad \text{[4.16]}$$

Where,

$$F = 1-\alpha-\gamma+\alpha\left(1+\frac{[S]}{\alpha K_S}\right)^4+\gamma\left(1+\frac{[I]}{\gamma K_I}\right)^4+\frac{[I][S]}{K_I K_S}\left\{\frac{6}{\alpha}+\frac{6}{\lambda}+\frac{3[I]}{\gamma K_I}\left(\frac{3}{\alpha}+\frac{1}{\gamma}\right)\right.$$

$$\left.+\frac{[I]^2}{2(\gamma K_I)^2}\left(\frac{7}{\alpha}+\frac{1}{\gamma}\right)+\frac{3[I][S]}{\gamma K_I \alpha K_S}\left(\frac{1}{\alpha}+\frac{1}{\gamma}\right)+\frac{[S]^2}{2(\alpha K_S)^2}\left(\frac{7}{\gamma}+\frac{1}{\alpha}\right)\right\} \qquad \text{[4.17]}$$

Specifically, at a low inhibitor and substrate level, higher order items are negligible, therefore, equation [4.16] can be simplified to

$$r = 4kE\frac{[S]\left\{1+\frac{3\beta_{IS}[I]}{2K_I}\left(\frac{1}{\gamma}+\frac{1}{\alpha}\right)\right\}}{K_S+4[S]+\frac{4K_S[I]}{K_I}} \qquad \text{[4.18]}$$

Equation [4.18] is mathematically similar to the binding model of Alberty [105, 114,

245]. This explains the applicability of the conventional binding model of Alberty for LDH, under the low substrate and inhibitor condition.

At low inhibitor and moderate substrate concentrations, [*I*] items are negligible, then equation [4.16] can be simplified to

$$r = \frac{4kE[S]}{K_S} \frac{1 - \beta_S + \beta_S \left(1 + \frac{[S]}{\alpha K_S}\right)^3}{1 - \alpha + \alpha \left(1 + \frac{[S]}{\alpha K_S}\right)^4} \qquad [4.19]$$

Equation [4.19] is identical to equation [10], which demonstrates that equation [4.16] is a more general equation for the initial rate of LDH catalyzed reactions.

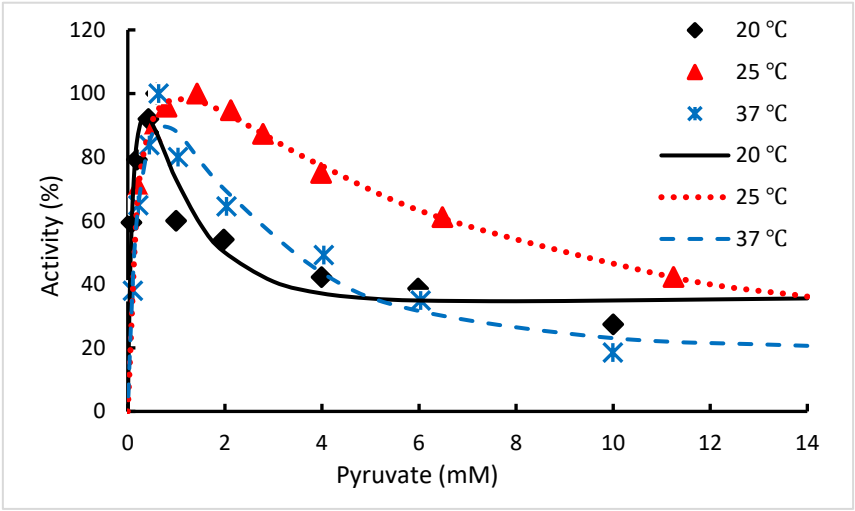
4.5.Discussion

4.5.1. Substrate concentration and temperature effects

To study the substrate concentration and temperature effects, human LDH type 1 (hLDH-1) was used as an example. Three data sets were selected from two publications, reported by Vesell [248] and Gubernieva [249], for the initial catalytic rates of hLDH-1 in converting pyruvate to lactate. The isoenzymes were extracted from human heart, psoas muscle, liver, or pancreas tissue within 12 hours of death. The experiment was controlled at 20, 25, and 37 °C, respectively. The isoenzymes were pre-saturated by NADH before initial rates were measured. The initial reaction rates were measured with different levels of pyruvate by a spectrometer at 340 nm. The data were selected from these publications and reanalyzed via equation [4.10].

Enzyme hLDH-1 exhibited sensitive responses to temperature (Fig. 4-5). This isoenzyme reported significant substrate inhibition when pyruvate concentration is higher than 1 mM. The calculated $\beta < 1$ indicates that the subsequent substrate binding reduced the reactivity. Thus, a higher ratio of $[ES_i]/E$ ($i > 1$) could lower LDH activity at a high pyruvate concentration. A smaller α indicates enhanced binding on subsequent sites. Therefore, at 37 °C, reporting small α and β values, could be the preferable choice for suppressing the activity of hLDH-1.

A



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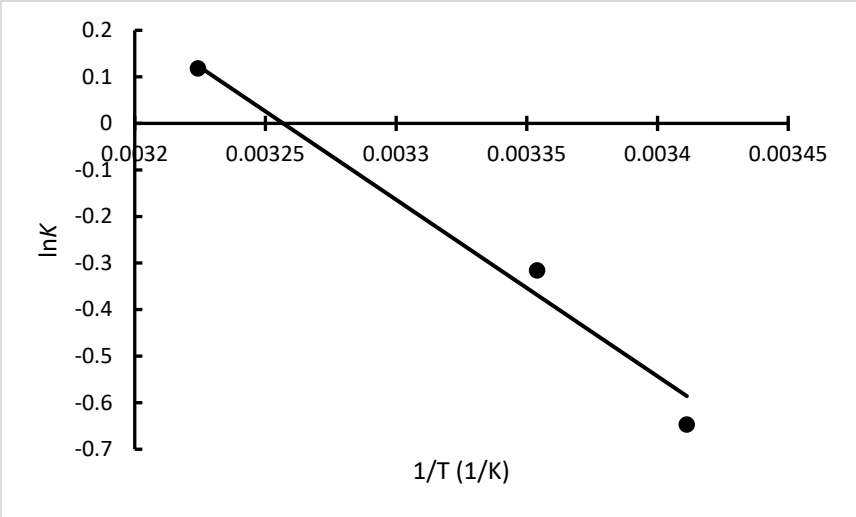


Temp. (°C)	kE/r_{max}	α	K (M)	β	αK (M)
20	1.6	2.9	0.52	0.062	1.5
25	1.3	14	0.73	0.047	10
37	1.5	3.0	1.1	0.034	3.4

Fig. 4-5. (A) Temperature effect on the activity of hLDH-1 with data taken from publications [248, 249]; (B) Van't Hoff Equation simulation of hLDH-1 K values. hLDH-1 kinetic parameters at different reaction conditions are listed in the table. The dots denote the experimental data and the lines represent the simulation results.

Equation [4.10] can be applied to predict the substrate concentration when the activity reaches peak value. If we let

$$\frac{dr}{d[S]} = 0 \qquad [4.20]$$

the theoretical substrate concentrations to reach the peak activity can be calculated as 0.4, 1.1 and 0.7 mM at 20, 25, and 37 °C, respectively, which agree with observed values in the experiments (Fig. 4-5).

Besides explaining the interactive properties and simulating the kinetic observations, equation [4.10] helped to reach a comprehensive understanding of temperature on pyruvate binding, which was not reported elsewhere. For a robust enzyme, if its structure is not sensitive to temperature changes, the binding saturation coefficient should approximately follow the Van't Hoff Equation [250] as

$$K = \frac{k_-}{k_+} = \frac{k_{0-} \exp\left(-\frac{E_{a-}}{RT}\right)}{k_{0+} \exp\left(-\frac{E_{a+}}{RT}\right)} = K_0 \exp\left(-\frac{\Delta E_a}{RT}\right) \qquad [4.21]$$

Where, ΔE_a is the activation energy difference between the backward and forward reaction or the heat of binding, R is the ideal gas constant, T is temperature (K); K_0 is a constant.

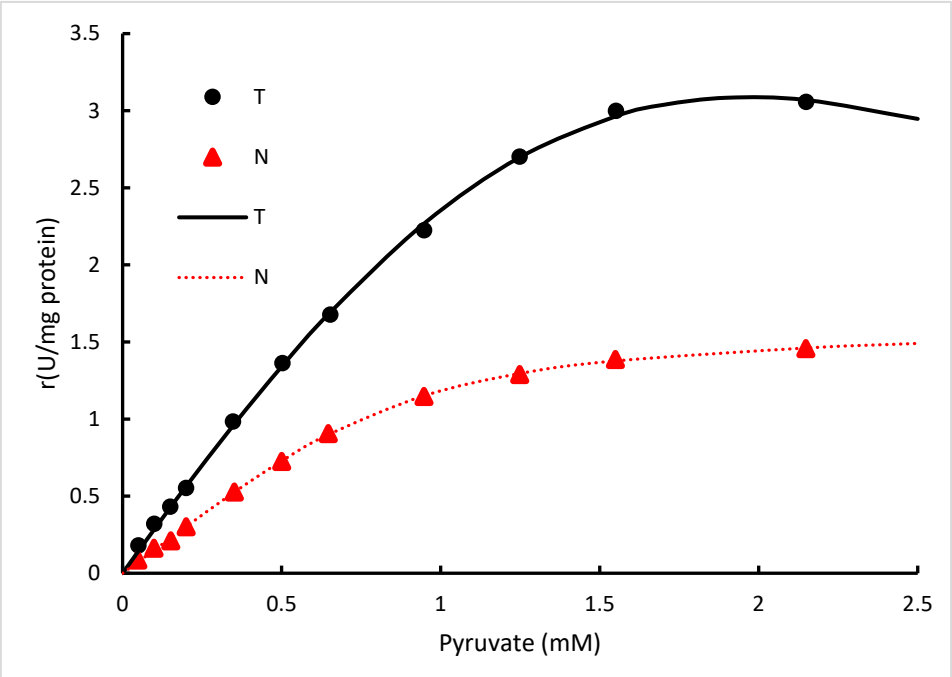
The approximately linear regression indicates that hLDH-1 is a relatively robust enzyme within the studied temperature range of 20 ~ 37 °C (Fig. 4-5 B). The calculated ΔE_a was 31.5 kJ/mol. The relatively integral enzyme structure indicates that the hLDH-1 subunits are relatively rigid. The initial binding affinity at other temperatures within the studied temperature range can thus be predicted via this model.

4.5.2. Cancer diagnosis

Cancer is one of the global leading causes of death [251]. Abnormal LDH kinetic properties and concentrations have been observed for tumor cells in comparison to normal cells [252, 253]. Currently, LDH is a useful marker for diagnosing cancer due to its role in the final step of the aerobic glycolysis [254].

One set of data from experiments conducted by Talaiezadeh. [91] was reprocessed with current model to provide insight on its behavior. In [91], LDH isoenzymes were extracted and purified from normal and malignant human breast tissues. The enzymes were dissolved in a pH 8 buffer solution. The experiments were conducted similarly to those used in section 4.5.1.

Fig. 4-6 showed that LDH from tumor human breast cells (T-hLDH) exhibited an enhanced catalytic activity as compared to the isoenzyme from normal human breast cells (N-hLDH). This is confirmed by the k values of T-LDH which was five-fold larger than that of N-LDH. The small α values of both LDH isoenzymes indicate that subsequent substrate binding was favored by the cooperative effect. However, this cooperative effect inhibited the enzymatic activity, indicated by the $\beta < 1$. The smaller β value of T-LDH than that of N-LDH indicates that T-LDH is a more substrate sensitive isoenzyme than N-LDH. Since dynamic intracellular pyruvate concentration always remains low in mammalian cells [31, 255], a quantitative analysis of k/K showed that the reaction within tumor cells could be potentially two-fold of that in normal cells at very low pyruvate concentrations (Fig. 4-6). This explains the abnormal high glycolysis metabolic activity observed in tumor cells [256, 257].



Source	k (mU/mg protein)	α	K (mM)	β	k/K (U/M·mg protein)
Normal human cells	4.7	0.056	13	0.10	0.36
Tumor human cells	27	0.050	37	0	0.73

Fig. 4-6. The initial rates of LDH from tumor human breast cells (T) and normal human breast cells (N) vary with substrate concentrations in conversion of pyruvate to lactate; The data were taken from the publication [91]; Kinetic parameters of different LDH isoenzymes are listed in the table. The dots denote the experimental data and the lines represent the simulation results.

While N-LDH still retained a reduced activity ($\beta = 0.1$), T-LDH became inactive ($\beta = 0$) when more than one substrate molecule was bound on it. Therefore, molecules which have similar structures as pyruvate may strongly inhibit T-LDH activity, while have lesser effects on N-LDH.

4.5.3. Inhibitor evaluation and dosage prediction

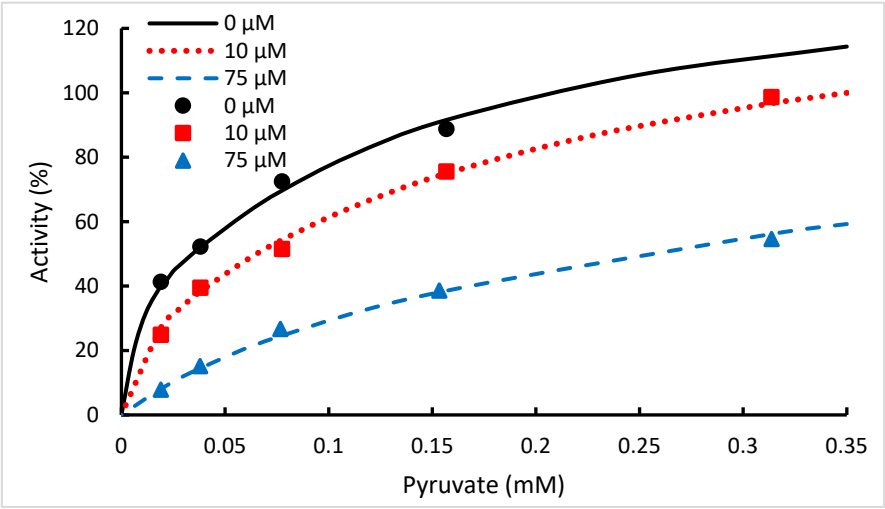
Because most tumor cells heavily rely on an enhanced glycolysis activity in converting glucose to lactate to maintain metabolic requirements [258], studies have been focused on those inhibitors which can significantly reduce LDH activity for potential cancer treatment [259, 260].

The experiment data were reported by Rodriguez-Paez [240]. Initial rates of pyruvate conversion to lactate catalyzed by mouse LDH were measured with different additions of N-propyl oxamate. The experiments were conducted similarly as the case studies utilized in the previous sections.

The data were reprocessed via equation [4.16] and shown in Fig. 4-7. One set of parameters was able to elucidate the kinetics at various inhibitor and substrate conditions. Thus, quantitative standards can be built up to characterize the effect of an inhibitor regardless of its dosage. Typically, small γ, K_I, and β_{IS} values, which indicate high binding competitiveness and reduced reactivity, are preferred for a high performance inhibitor. In this case study, the calculated α, $\gamma > 1$ indicates that both pyruvate and N-propyl oxamate are retarding ligands, inhibiting subsequent substrate and inhibitor binding on the enzyme. N-propyl Oxamate exhibited lower binding affinity than pyruvate at low concentrations ($K_I > K_S$), while its binding affinity

exceeded pyruvate at high concentrations ($\gamma K_I < \alpha K_S$). Substrate inhibition was not observed from this experiment ($\beta_S = 1.0$). However, when both substrate and inhibitor molecules were bound, the LDH activity could be reduced by the substrate-inhibitor cooperation ($\beta_{IS} = 0.7$). Therefore, oxamate inhibited LDH activity not only by seizing the substrate binding domains but also by reducing the reactivity. This makes N-propyl oxamate a qualified inhibitor reducing LDH activity within wide substrate concentration ranges.

A



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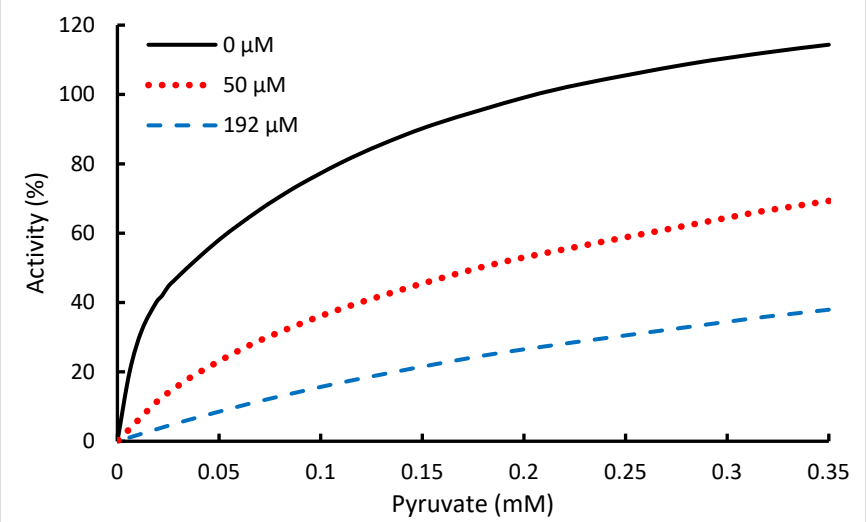

kE/r_{max}	α	γ	β_S	β_{IS}	K_S (mM)	K_I (mM)	αK_S (mM)	γK_I (mM)
30	38	7.3	1.0	0.70	2.5×10^{-3}	3.6×10^{-3}	0.095	0.026

Fig. 4-7. (A) Inhibition of N-propyl oxamate on LDH for the conversion of pyruvate to lactate with experimental data taken from the publication [240]. (B) Kinetic prediction of N-propyl oxamate required to inhibit LDH activity at 50 % and 25 % at 0.2 mM pyruvate concentration. The dots denote the experimental data and the lines represent the computing results.

Besides the mechanistic understanding of the enzyme properties, this model also serves for adequate inhibitor dosage study. An accurate inhibitor dosage to reach a target LDH activity can be predicted via this model instead of testing in the lab. For instance, based on the disease diagnosis study (Fig. 4-6), The enzymatic activity within a normal cell is 50 % of a tumor cell around the pyruvate concentration of 0.2 mM which is a typical level within cells [261]. Via equation [4.16], it can be calculated that 50 μM of N-propyl oxamate can successfully halve the activity of LDH, while 192 μM can further halve it (Fig. 4-7 B). This provides the basis to set up adequate inhibitor or drug doses, avoiding any insufficient or excessive dosage.

Overall, this model builds up uniform standards to evaluate different kinds of inhibitors by comparing γ, K_I, and β_{IS} values. It is able to use one set of parameters to mechanistically evaluate the inhibitors regardless of their concentrations, instead of using classical M-M model to get different sets of kinetic parameters at various inhibitor levels. Besides, it can compute a theoretical inhibitor dosage to reach a target inhibitory effect, which may reduce the pharmaceutical research cost during the clinical or preclinical stage.

4.6.Conclusions

In this chapter, a new mechanistic kinetic model was developed to explain the kinetic properties and dynamic behaviors of oligomeric enzymes, such as LDH, based on the interactive nature of enzyme-ligand bindings. The effects of temperature, substrate concentrations, and competitive effectors were analyzed mechanistically with

case studies. The kinetic information extracted from the new models provided a theoretical understanding of LDH, and was able to evaluate inhibitor effect with uniform standards and predict the kinetics at different substrate and inhibitor levels. This may promote further advancements in disease diagnosis, enzyme activity control, drug evaluation as well as adequate medication dosage.

Based on the above two chapters, the kinetic modeling methods were built up to achieve the high cell culture titer and understand the enzyme properties. A next chapter will focus on the biologics quality control by process development, which is also a crucial aspect in the biopharmaceutical industry.

Chapter 5

Monoclonal Antibody Quality Control by Process Development

5.1. **Abstract**

With the consensus that quality is always paramount in the biopharmaceutical industry, much attention has been paid on the quality control strategies. Process development is the bridge connecting the drug discovery and manufacturing, which has a crucial impact on the biologics quality. In this review, the process development tasks were briefly summarized; the critical quality attributes and quality control methods via process development were discussed. It aims at providing an overview picture of monoclonal antibody critical quality control strategies by process development based on the Quality by Design principle.

Keywords: Quality by Design; process development; critical quality attributes

Abbreviations:

CHO, Chinese Hamster Ovary; **CQA**, critical quality attributes; **DoE**, Design of Experiment; **HCP**, host cell proteins; **QbD**, Quality by Design

5.2.Introduction

After a fast expansion of monoclonal antibodies (mAbs) market, both the supply and demand of biologics are increasing rapidly [3, 6, 262]. Being life related, mAbs direct impact on the clients' health. Thus, the quality has been recognized as the most important aspect. Based on the Quality by Design (QbD) principle proposed by FDA, the quality of biologics and drugs should be well designed and developed in the pre-clinical stage instead of measuring during the manufacturing [263]. Therefore, a good understanding of the impact of different processes on quality is beneficial for mAb quality design and control.

Four stages were included in a typical process development project: cell line development, upstream process (cell culture) development, downstream process (purification) development and formulation development [264-266]. While cell line development may be excluded in some biopharmaceutical companies, it is discussed as one process development stage in this work. Critical quality attributes, including the amino acid sequences [267], N-glycan [268], charge variants [269], etc. have important impacts on the mAb efficacy and stability. Some of the quality attributes can be designed and controlled via multiple process development steps, while others can only be controlled at one certain unit operation. An understanding of the practical process development strategies for quality control can help to design a time- and cost-efficient manufacturing process to achieve the desired mAb quality.

In this chapter, the tasks of different process development steps were summarized; the concept of Quality by Design (QbD) was introduced; the process development

strategies for quality control were discussed. This work aims at providing an overview picture of quality control strategies by process development in the biopharmaceutical industry.

5.3.Process Development

Shown in Fig. 5-1, four process development stages are classified by their working tasks. The full length of a process development project may take one to three years, depending on the requirements of the project and the local regulations. Obviously, a long period of process development is beneficial to develop a high quality manufacturing technology, while short period is always desired by the biopharmaceutical companies to reduce the cost and accelerate the product commercialization process [267]. A practical process development strategy should be a good balance of the above two concerns.

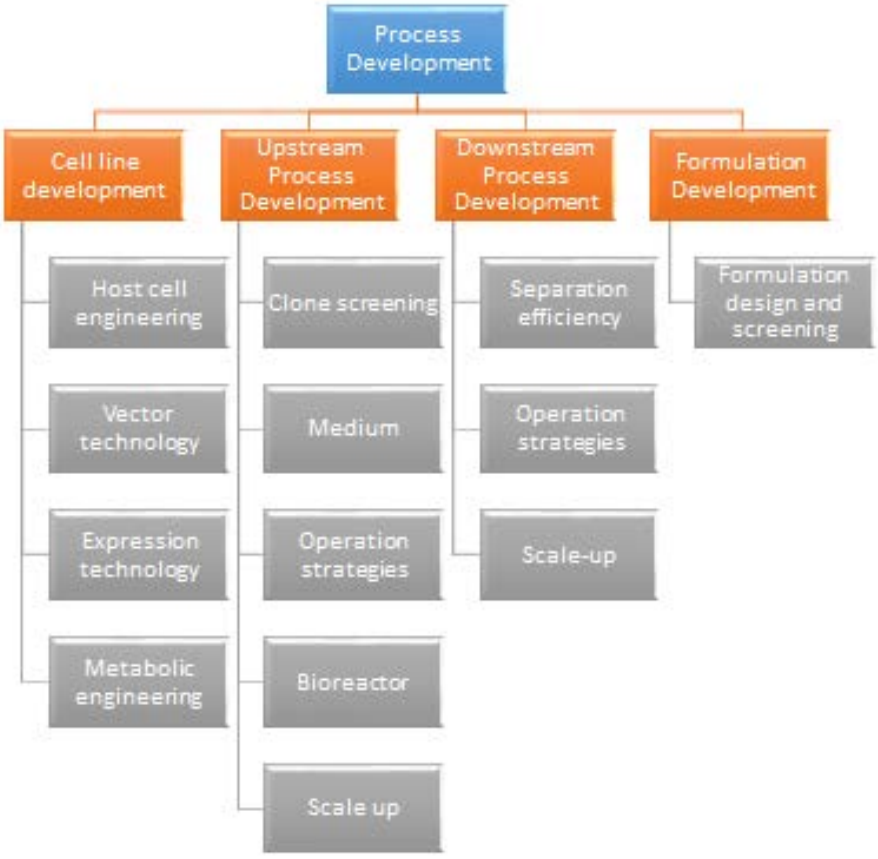

Fig. 5-1. The tasks of each process development stage.

5.3.1. Cell line development

The core task of cell line development is to develop monoclonal host cell line candidates with stable, high titer expression of high quality mAbs [16, 270]. As the first stage of process development, the result of cell line development has a sustained impact on the following stages. After the gene editing and vector transfer to the host cells, around 10,000 clones are screened to get a few high performance monoclonal cell line candidates [270]. They are then sent to upstream for a further screening and cell culture development. Series of characterization need to be conducted to ensure that the cell lines are free of mycoplasma and virus, as well as the therapeutic protein has the

correct structure and amino sequence. An early detection of titer and protein quality is also taken to provide hints for upstream process development. After the determination of top clones, the clone stability test will be done to ensure good stability of the selected clones. Meanwhile, the master and working cell banks are established.

5.3.2. Upstream process development

Upstream process development focuses on cell culture development [264]. It aims at developing a scale-up-able and robust process to reach high titer and high quality [42]. As the second process development stage, upstream usually starts with a few top clone candidates. At the early upstream stage, shake flasks are widely used for an overall understanding of the cell culture properties and a rapid approach to a suitable cell culture process. While at the late upstream stage, bioreactors are more commonly utilized to better control and accurately regulate the cell culture parameters, and then further scale up to the pilot production system.

5.3.3. Downstream process development

Downstream process development is responsible to develop a scale-up-able and robust process to achieve high yield, high purity and high quality products [271]. As the third process development step, it starts once receiving the harvest samples from upstream. Different chromatography and associated devices are used to capture the target mAb, remove impurities and control the quality attributes. Various buffers, columns and process parameters are optimized at this stage based on the Design of Experiment (DoE) principle. At current technology level, > 90 % of purification yield can usually be reached for each unit operation, with a total downstream yield > 60 %

in the industry.

5.3.4. Formulation development

As the last stage of process development, formulation development is to achieve a stable and appropriate formulation buffer to ensure the biologics are easy and safe for patient to take [272]. Typical formulation may contain salts, carbohydrates, amino acids, surfactants, etc. Compared to the other three stages, formulation development pays more attention to keeping the intellectual rights and developing a platform formulation.

Fig. 5-2 provides an example of process development framework. It keeps similar when being scaled up to a manufacturing system. The usage and arrangement of the columns may vary depending on the nature of the project. Each step plays its unique role for quality control, which will be further discussed.

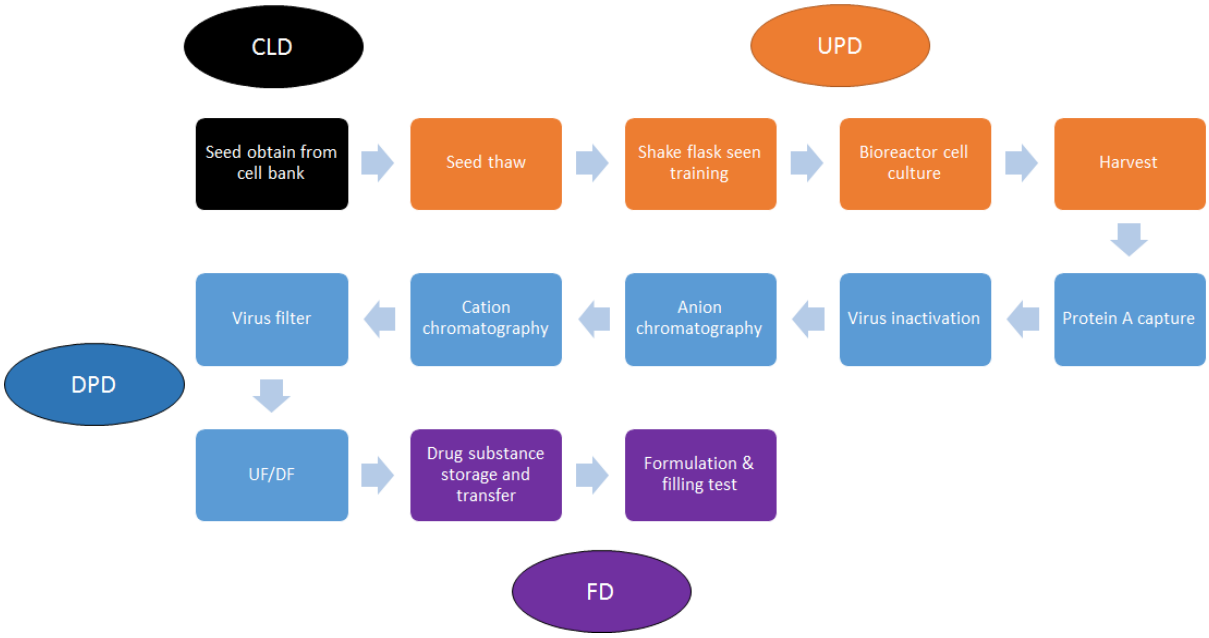

Fig. 5-2. One typical flow chat of a typical bio-process development operation process from host cell obtain from cell bank to the formulation and filling. CLD: cell line development; UPD upstream process development; DPD, downstream process development; FD, formulation development.

5.4. Quality by Design

The concept of QbD can be traced back to 2003, proposed by FDA, and be further officially included in the FDA Process Validation Guidance in 2011[263, 273]. The same concept was explained in ICH section Q8 as "A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management"[274]. It means products and process characteristics are scientifically designed to meet specific objectives, not merely derived from empirical knowledge or performance of test batches[263]. Thus, a mechanistic understanding of the process

performance, as well as the quality attributes mechanism and effect, is indispensable to develop QbD based bioprocesses. Bio-process development, from the above point of view is no more solely engineering work. It needs a scientific understanding of the molecular biology, enzymology, genomics, bio-kinetics, and fluid mechanics, etc. An interdisciplinary education or knowledge background may be a requirement for an outstanding PD scientist in the near future.

Generally, three aspects are included in the QbD concept[273, 274]:

A). Process Development. It aims at design and develop a good process to meet the quality attributes requirement;

B). Process Characterization. It targets on building up a robust and risk managed process;

C). Process Validation. Its goal is to set up validated design space.

In this chapter, our discussion is focused on (A) process development, which is the first and fundamental part to mechanistically design and control the quality attributes.

5.5. Critical Quality Attributes Control

A good understanding of these critical quality attributes (CQAs) and the quality control process development principles will be beneficial to develop an appropriate process for mAbs manufacturing. Table 5-1 summarized the ability of each process development stage or unit operation to regulate the CQAs, which will be discussed in this section.

Table 5-1. Typical critical quality attributes control strategies at different process development steps or unit operations.

PD	Strategies/ Unit Operation	Amino Acid sequence	N-Glycan	Aggregation	Charge Variants	Fragments	Other Impurities
CLD	• Cell Engineering	√√	√		√	√	
	• Clone screening	√	√		√		
UPD	• Top clone screening		√√		√		
	• Medium optimization		√√	√	√√	√√	
	• Process parameter optimization		√√	√	√√	√√	
DPD	• Protein A					√	√√
	• CEX				√√		√
	• AEX						√
	• HIC			√√			√
FD	• Formula optimization						

'√' indicates the quality attribute can be controlled by the corresponding strategy. More

‘√√’ means this step is undertakes the major responsibility to control this quality attribute. CEX, cation exchange chromatography; AEX, anion exchange chromatography, HIC, hydrophobic interaction chromatography

5.5.1. Amino acid sequence

Amino acid sequence is the primary structure of an mAb. The occurrence of amino acid sequence mutation has a huge impact on the mAb efficacy and stability [275, 276], thus it should be detected as early as possible. Peptide mapping is an essential technique to detect and monitor single amino acid sequence mutation. The principle behind peptide mapping is: if two proteins have the same primary structures, then cleavage of each protein with a specific protease or chemical cleavage reagent will yield identical peptide fragments. However, if the proteins have different primary structures, and the cleavage will generate unrelated peptides [277, 278].

Though it should be well designed in the new drug discovery stage, the mutation may occur during the cell line development stage. It is possible due to the operation of gene transfer to the host cells, the impact of the selection pressure or the instability of the host cell or the gene itself. If the root cause is because of the gene instability, the mAb candidate may need to be turned back to the new drug discovery stage for a better molecule design. If the mutation occurs because of the cell engineering operation, either changing the selection pressure or rescreening the clones should be taken to solve this problem.

5.5.2. N-Glycan

N-terminal amino acid linked glycosylation is the main type of glycosylation for mAbs. The distribution of the modified mAb molecules is named as N-glycan. N-glycan may impact on the ADCC activity, in-vivo half-time, etc. [268, 279]. One straightforward method to control N-glycan is modifying and screening the host cells at the cell line development stage. Significant N-glycan difference (> 25 %) has been reported among various host cell types [16, 20, 270, 279]. This gap is usually difficult to be filled via cell culture. After the host cell line is determined, N-glycan can be further regulated during cell culture via medium and process parameter optimization.

By adding or removing certain chemicals, such as amino acids, manganese ions, uracil, and galactose, etc., glycan related metabolic pathways can be directly activated, inhibited or changed [268, 280]. Meanwhile, cell culture parameter optimization is a mild method to fine-tune the N-glycan. Process parameters, such as temperature, pH, and dissolved oxygen etc., can indirectly impact on the intracellular enzymatic activities and pathways, and the influence will be reflected by the N-glycan [215, 268, 281].

In the industry, cell line and clone screening is the most effective way to significantly regulate the N-glycan. Medium and cell culture parameter optimization would be the next step to accurately control N-glycan, especially for biosimilar products. Matching the glycan profile at early stage is always preferred than doing that in the late stages.

5.5.3. Aggregation

Protein molecules tend to place the hydrophilic chains into the aqueous solution while orientate the hydrophobic chains into a hydrophobic microenvironment [282].

This means proteins may form colloids which is observed as aggregation. Aggregation negatively impacts on the drug stability and efficacy.

To reduce the occurrence of aggregation, dispersants can be added into the bioreactor during the cell culture process, which should be controlled at a reasonable low level because they are toxic to the cells [282]. During the downstream process, hydrophobic interaction chromatography can be applied [283]. Typically, the aggregation will be < 1% after this downstream unit operation [264].

5.5.4. Charge Variants

Charge variants are one profile indicates the distribution of the charged mAb molecules. Variation of charge variants leads to the efficacy instability [284]. Usually, high contents of neutral mAb molecules from batch to batch are preferred. Constant distribution of change variants are required to maintain the quality consistency.

Bioflavonols, Trace elements, and vitamins are able to impact the charge variants during the formation of biologics. By optimizing their contents in the medium, the charge variants can be regulated during the cell culture process [285]. Meanwhile, cell culture time, pH, and temperature are also influential parameters [207, 269]. During the downstream process development, cation exchange chromatography is the major method to control this quality attribute by removing certain abundant charged mAb fractions. This is an alternative choice when the charge variants are not able to be well controlled by cell culture, because this downstream operation may lead to a significant yield loss [284, 286].

5.5.5. Fragmentation

Fragmentation refers to mAb molecules losing part of its structure, which is exhibited as low molecular weight. [287] They usually occur during the cell culture. [288] Fragment profiles will impact on the stability and in-vivo half-time of the biologics. Most of the fragmentation mechanisms can be grouped into two types:

A). Breaking of the disulfide bond(s). It usually yields to free light chain, heavy chain and half-mer. [289, 290]

B). Breaking of peptide bond(s). Various types of fragments may be observed depending on the broken peptide bonds.[291] Typical fractured bonds include Asp-Gly [292], Asp-Pro [293], Asp-Asp [294], Gly-Thr [295], Gly-Ser [295], Cys-Cys [296], Asn-Ser [297], etc.

To repair the fragments, the fragment mechanism should be confirmed first. If it is because of the breaking of the disulfide bond(s), mild oxidizer should be applied to re-oxidize the free thiol to disulfide. If it is because of breaking of peptide bond(s), solutions may be to add enzymes, adjust pH, etc. However, the fragmentation issue is difficult to be solved in reality once it occurs, due to the complexity of the fragment profile. The fragments may exhibit similar affinity, molecular size and charge variants compared to the intact mAb. There are no Protein A, hydrophobic interaction chromatography or ion exchange chromatography that is perfect to remove different kinds of fragments. [287, 289, 290, 298] Therefore, it is desired to minimize the fragment formation via cell culture and the harvest operation.[299]

5.5.6. Other Impurities

Purity is the basic and critical requirement for mAbs. While upstream produces

numerous by-products simultaneously with the target mAb, downstream bears the task to remove all the impurities.[287] Other than the above discussed CQAs, other impurities include host cell proteins (HCP), DNA, leached materials from previous unit operations, medium components, endotoxins and virus. [276] Protein A chromatography is one of the most important unit operation for highly efficient mAb capture and impurities removing. It distinguishes itself with high selectivity on IgG type antibodies. [283] During this step, most HCP, DNA, medium components and virus particles are removed. [300, 301] The major disadvantage of Protein A is the high cost. In the industry, multiple cycles are applied to the Protein A unit operation to improve the utilization efficiency.[283, 300]

Directly after the protein A, ion exchange chromatography is often used to further remove the remaining HCP, residual DNA, leached protein A materials, medium components, endotoxins and virus.[302, 303] Hydrophobic interaction chromatography is then used as a complementary to remove most aggregates. [283]

5.6. Challenges and Trends

Process development is the bridge to achieve high quality products in manufacturing. Challenges exist to meet the increasing demands of high quality biologics, including:

A). Scale-up stability. Though, a couple of scale-up strategies have been built up during the recent decade, it is not rare to observe quality attribute variation when the process is scaled up to a pilot (250 – 1,000 L) or full (2,000 – 25,000 L) scale production

system, especially in the upstream due to the involvement of cells.

B). Lack of efficient methods to solve some quality problems. In the upstream, a single medium component or operation parameter change may impact on multiple metabolic pathways. Thus, it is not easy to accurately regulate one single quality attribute without influencing others. Meanwhile, downstream has few methods to polish N-glycan profile, or recover the fragments to intact mAbs.

C). New and validated processes to meet the recent perfusion and single-use technologies. A perfusion bioreactor operation may last up to three months for continuous upstream and downstream production, which requires higher product quality stability than the current fed-batch bioreactor operations. Single-use bioreactors are good for rapid production and risk control of sterilization. However, leachables and extractables from single-use bioreactor construction materials need to be well validated before utilization. In the downstream, it is difficult to implement based solely on sing-use bioreactors due to the high expense of the columns.

5.7. Conclusion

The demands on high quality biologics will continue to increase in the coming decades. Process development takes the responsibility to set up technologies for high quality biologics manufacturing. In this chapter, the tasks of different process development stages were briefly reviewed. The concept of QbD and process development strategies for quality control were summarized. Continuous input is needed to build up scale-up-able and robust plat-form technology to manufacture high

quality biologics.

Chapter 6

Summary and Outlook

Impacted by the continuous progress of medical insurance system, drug manufacturing technology and new drug discovery all over the world, much attention has been attracted to the biopharmaceutical industry globally. Rapid and accurate methods for biologics screening are process development are highly demanded to ensure efficient and successful biopharmaceutical research and commercialization.

Kinetic modeling, as discussed, is a potential method to meet the above requirements. It can be applied for biologics and indication screening, process development and elucidating of enzyme kinetic properties, which can serve the biopharmaceutical development from different aspects. In this dissertation, all of following aspects were discussed:

A) The application of kinetic modeling for biologics and indication screening;

B) A novel method for rapid temperature shift strategy screening to reach high titer via kinetic modeling;

C) A mechanistic elucidating of the kinetic properties of lactate dehydrogenase. This can be used for explanation the enzyme performance and prediction of appropriate drug dosage.

D) Brief introduction of mammalian cell culture and biologics quality control strategies

Though kinetic modeling is not mandatory under the current regulation, there's no doubt, well developed models explaining the kinetic data would be a bonus to get the IND approval. In the Chinese Pharmacopoeia (2015), kinetic modeling is mandatory required to explain and predict the thermal and storage stability. It will not be a surprise if this modeling strategy is required for other pre-clinical researches in the near future.

Limited by the research time, this dissertation may not have covered all the aspects of kinetic modeling research in the biopharmaceutical industry. A further study can continue based on the following suggestions:

A) Reducing the time and experiment amount for kinetic modeling calculation with the prediction accuracy improved;
B) Modeling for therapeutic protein quality understanding and control;
C) Modeling for perfusion cell culture and continuous protein purification process for developing the next generation manufacturing technology;
D) Building up kinetic modeling method for scale-up/-down;
E) Further application of mechanistic kinetic modeling strategy to elucidate crucial oligomeric enzymes to better understand cancer mechanism and dosage control strategies.

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Educational Background

State University of New York – ESF	University of Hawaii at Manoa	Peking University
PhD Candidate	Master	Bachelors
Bioprocess Engineering	Mechanical Engineering	Chemistry Economics

Working Experience

Mab-Venture Biopharm. Co., Ltd (03/2017 – 12/2017), China
Sr. Manager (CMO site) & Principal Scientist (CRO site)

- Managing the department of upstream process development
- Leading project tech-transfer
- IND writing and submission of two projects to CFDA
- Participating in the perfusion system set up
- Supporting the design and building of the CMO site

Bristol-Myers-Squibb (01/2016 – 06/2016, 01/2018 – Now), US
Upstream Co-op

- Cell culture process development
- Temperature shift strategy modeling
- Kinetic modeling of lactate dehydrogenase

Downstream Co-op

- Biologics purification process development
- LMW mechanism and solution study

Yoation Printing and Dyeing Machinery Co. Ltd (08/2013 – 08/2014), China
International Marketing Manager (Department Leader)

- Led the international BD affaires, focused on European and Southeast Asian Market
- Supported the design and building of the manufacturing site Led technology patent applications

Social Activities

- SUNY-ESF (08/2015 – Now), US

Graduate Representative of Safety Committee

- Chinese Antibody Society (05/2017 – Now), US & China

Member

Research & Study Experience

State University of New York - ESF (09/2014 – Now), US
Research Assistant

- Kinetic modeling and its application in the biopharmaceutical industry
- Oligomeric enzyme allostery and inhibition study

University of Hawaii at Manoa (08/2011 – 11/2013), US
Research Assistant

- Conversion of bagasse to levulinic acid with reduced waste and pollution (Supported by Sugar King Co. Ltd)
- Kinetic study of deactivation and activation of heterogeneous catalysts

Peking University (03/2009 – 07/2011), China
Research Assistant

- Chemical synthesis and self-assembly study of surfactants (Supported by PetroChina Company)
- Study of the interaction of surfactants and bio-molecules

Publications

1. **Peifeng Tang** and Jian Yu, "Kinetic Analysis of the Deactivation of a Heterogeneous Catalyst in Conversion Sucrose to Levulinic Acid", Industrial and Engineering Chemistry. July, 2014
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