OPTIMIZATION OF CLONE SELECTION PLATFORM FOR PRODUCTION OF RECOMBINANT PROTEINS

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Abstract

Background
Biologics are type of proteins that can be produced by expression in a foreign host for medical healthcare applications in humans. Monoclonal antibodies (mAbs) are type of biologics that are designed to specifically bind to one target, with a purpose to inhibit a certain molecule that could be associated with a disease process. Usually, the usage of mammalian cells as expression hosts are generally preferred for biologics production. Among the available choices of mammalian host cells, Chinese hamster ovary (CHO) cell line is considered to be the initial choice for biologics production due to their compatibility with human proteins and their scalability to grow in large scale.

Aims
This project aims to establish a clone selection platform, to identify the best producing clone from a specific cell line and its clones from metabolic point of view (CHO-SDI-X, CHO-SDI-Y, CHO-SDI-Z). These cell lines were established through site-directed integration. Specifically, the goal of the project is to:

- evaluate cell culture kinetics and metabolic profiles for each cell line in batch and fed-batch culture.
- identify the most adequate feed rate for future clone selection platform based on these cell lines and fed-batch regime.

Methods/Materials
Cells were thawed and passaged (seed trains) initially (target initial and end viable cell densities for each seed-train step were 0.5(±0.1) and 4 (±1) MC/mL, respectively), before cultured into batch and fed-batch mode. Secondly, cells were grown in batch mode and each cell line were grown in three biological replicates. Finally, during fed-batch mode, cell specific feed rate strategy was implemented as the feed strategy for the experiment. In addition, 3 different feed rates (0.5, 0.75 and 1.125 pL/cell/day) were tested to identify the adequate one for the cells in addition to measuring the produced metabolites. Samples were taken daily from each culture flask in both batch and fed-batch to measure viable cell density, viability, and metabolites (Gluc, Lac, Gln, Glu, NH4+).

Results
The data generated from the batch and fed-batch culture revealed differences in the cell kinetics and metabolic profile between CHO-SDI-Z and the other two cell lines. Also, during batch experiment, different growth phases of each cell line including the exponential growth phase was identified. In addition, after screening different feed rates and testing the cells in fed-batch mode it was identified that 0.75 pL/cell/day
was the most adequate feed rate, and 1.125 pL/cell/day drove the cells to die/crash suddenly after 12 days of continuous growing.

**Conclusions:**

As a conclusion, the starting point of fed-batch regime were determined for each tested cell line. Also, the driving factors for the end of the exponential phase and end of stationary phase have been identified. Furthermore, it was concluded that CHO-SDI-X and CHO-SDI-Y had similar cell culture kinetics and metabolic profiles, while CHO-SDI-Z had different demeanour. Finally, the optimal feed rate was identified.

**Keywords:** Biologics, recombinant proteins, CHO-cells, monoclonal antibodies, batch culture, fed-batch culture
**Popular Scientific Summary**

Biologics, which can be described as tiny “guided missiles”, can target specific molecules in the body. They are widely used in all treatments of various diseases, including rare disorders and cancer. Mainly, these specific drugs are designed to mimic the natural antibody that is produced in our body by immune cells, and it can be modified to recognize and bind to specific disease-causing molecules.

One of the popular cell lines in the field of biologics production is known to be Chinese hamster ovary (CHO) cells. These cells usually grown in suspension culture. There are three main approaches for bioproduction using this cell line including: batch, fed-batch and perfusion culture, differing by the way how nutrients and waste products are handled. Fed-batch is known to be the gold standard culture approach for biologics production, since it is more controlled than the batch culture environment, as well as being less sophisticated and time-consuming in comparison to perfusion culture.

The production of biologics in CHO cells is a complex process, it requires thorough expertise in molecular biology, cell culture techniques, and bioprocessing. However, it is a crucial method that allows for large-scale production of these therapeutic proteins, helping to provide effective treatments for various diseases and improving patients' lives.

The main aim of the project was to evaluate three generated CHO cell line clones (CHO-SDI-X, CHO-SDI-Y, CHO-SDI-Z) that were modified genetically, to assess their productivity and cytotoxicity, by checking the cell’s kinetics and metabolic profile. Furthermore, a new cell specific feed strategy was being tested with three different feed rates (0.5, 0.75 and 1.125 pL/cell/day). After investigating the cell lines, we identified one cell line with distinctly different kinetics than the other two cell lines. Our findings suggest that the main reason behind that is the different nature of the molecule that this cell line expresses. In conclusion, our findings show that one cell line (CHO-SDI-Z) distinctly differs from other cell lines opposing to our main hypothesis stating that the three cell lines should show similar metabolic and kinetic profiles. The project is a part of the process development at Cytiva, which aims to develop and optimize a clone selection platform to choose the best producer engineered cell line.
**Abbreviations:**

ATP ➔ Adenosine triphosphate
CHO ➔ Chinese hamster ovary
CLD ➔ Cell line development
CMV ➔ Cytomegalovirus
dCas9 ➔ deactivated Cas9
DHFR ➔ Dihydrofolate reductase
eGFP ➔ Green fluorescence protein
FDA ➔ Food and Drug Administration
Gluc ➔ glucose
Gln ➔ glutamine
Glu ➔ glutamate
GOI ➔ gene of interest
GS ➔ Glutamine synthetase
HEK ➔ Human embryonic kidney
IgG ➔ Immunoglobulin G
Lac ➔ lactate
mAb ➔ Monoclonal antibody
MC/mL ➔ Million cell per milliliter
MSX ➔ methionine sulphoximine
MTX ➔ Methotrexate
NH4+ ➔ ammonia
PDT ➔ Population doubling time
pL ➔ Picolitre
POI ➔ Protein of interest
PPT ➔ Pentose phosphate shunt
qA ➔ Quality attributes
qP ➔ Specific metabolic rate (productivity/consumption)
rDNA ➔ recombinant DNA
RI ➔ Random integration
SDI ➔ Site-directed integration.
TCA ➔ Tricarboxylic acid
VCC ➔ Viable cell concentration
VCD ➔ Viable cell density
1. Introduction

1.1 Biological drugs

Since the discovery and approval of recombinant insulin in 1980s, around hundred or more new recombinant therapeutic proteins were approved by the US Food and Drug Administration (FDA) or the European Medicines Agency (1). Biological drugs, also known as biologics, are defined as drugs produced in expression host for human and animal healthcare applications, such as vaccines, drugs, and antibodies (2). After the emerging of biologics in the drug market, the biopharmaceutical industry has seen rapid growth with a pace of 10-20% annual increase in revenue worldwide (3).

Differences between biologics and synthetic drugs can be summarized in the nature of the product, the source of the active agent, structure, manufacturing methods and handling (4). Biologics are known to have much more complex structure and their quality attributes (qA) are highly dependent on starting material and production process (5). Due to a nature of their production process, biologics require more complex procedures and longer time frame for purification from the host cell molecules or any other impurities in order to be used in healthcare applications (4). Comparing to synthetic drugs, biologics are more sensitive to degradation in the alimentary system and have limited permeability through the intestinal epithelium. Therefore, biologics are rather administered via direct injection or infusion (6). All the above-mentioned differences reflect on a higher production and, subsequently, over-all per dose costs of biologics comparing to synthetic drugs.

1.1.1 Monoclonal antibodies

Monoclonal antibodies (mAbs) are man-made immunoglobulin (IgG) molecules that have mono-specificity to a certain molecule or antigen (6). Worldwide, around five hundred seventy therapeutic mAbs have been studied in clinical trials by commercial companies while at least seventy-nine of them were approved by the FDA (8). In general, mAbs consist of two light chains and two heavy chains (Error! Reference source not found.), at which both light and heavy chains contain variable and constant domains. The heavy chain contains 4 domains (Vh, Ch1, Ch2, Ch3) with a flexible hinge region. Light chain consists of two subdomains, VL and CL (6). In addition, there is the fragment antigen-binding region (Fab) which is the region on an antibody where antigens bind to, as well as the fragment crystallizable (Fc). Fc consists of paired hinge C_{H2}-C_{H3} domains which bind to various Fc receptors and blood proteins to activate the system for target neutralization (6).
mAb can mimic the function of natural occurring IgG by acting on a certain molecule, which could be implicated in a disease pathogenesis. However, their mode of action/effect can differ from inhibition, over degradation, to activity modulation (7,8,9).

1.1.2 Biologics’ production regimes/approaches

Usually in biopharmaceutical industries, growing cells as a suspension is more common than the adherent culture approach since in suspension culture one can grow cells in higher volumes, resulting in higher cell densities in comparison to adherent culture. In addition, suspension culture is a better controlled environment than the adherent culture. There are different production approaches/regimes that can be used for growing cell cultures: batch culture, fed-batch culture, perfusion culture (Error! Reference source not found.) (10). One of the cell suspension culture approaches is known as batch culture, which is considered as a closed system, where cells are supplemented with the necessary nutrients at the start of cultivation only, and thereafter left to grow until they die due to nutrient depletion (11).

In a fed-batch regime, which is considered as the common approach for biologics production, cells are supplemented with the necessary nutrients throughout a duration of the entire bioprocess. Such continuous supplementation of nutrients allows the cell cultures to grow to higher densities and achieve higher product quantities until the accumulation of secondary metabolites or waste products inhibit cells’ metabolic activities and lead to cell growth arrest or even cell death (12).
The perfusion culture, which is the most controlled approach among all the other approaches, is similar to the fed-batch when it comes to supplementing the cells with necessary nutrients, before starting the culture process and also during the experiment. But it differs from fed-batch, at which the secondary metabolites or waste products are eliminated from the system, because their accumulation could inhibit the cell growth and the production process. Although perfusion seems like a very controlled and clean system, it is a less preferred approach due to the high material consumption and the requirement for more skilful labour force to make the process work (13).

1.2 Cell line development and clone selection

In order to express the protein of interest (POI) which at the end of the process can be used as therapeutic agent or even for in-vivo research studies, an expression system is needed. Such system consists essentially of three different components that are crucial for appropriate bio-production of the recombinant protein of interest (14). First component that is needed is known as the biological environment, which usually represents a host cell that is responsible for providing energy and machinery for synthesizing the recombinant protein of interest. Second part is the vector of interest which facilitates the introduction of genetic material into a host cell. It contains necessary regulatory parts, which help in the replication of the genetic material, and usually a selection marker that allows the maintenance of a vector and selection of successfully transformed cells (15). The last component is an expression cassette, which contains the open reading frame for the protein of interest (16) and all the components necessary for its transcription and translation (17).
The process of cell line development (CLD) is a multi-step process: starting with the construction of the expression vector and the gene transfer to the biological host, moving to using the right selectable marker. Then cells are screened for high productivity and growth recovery and finally clone selection (18). Cell engineering and cloning is a sophisticated process. The process of selecting the most productive and stably cloned cell lines in a population of transfected engineered cells needs more hands-on work and time and is hence considered to be one of the major challenges facing the biologics industry (19).

Usually, mammalian expression vectors consist of one cassette of antibody genes and a selectable marker gene for expression in mammalian cells. For high expression of antibody, a strong promoter, such as cytomegalovirus (CMV) promoter, is used to drive the expression of both heavy and light chains (20). Moreover, a selectable marker is needed to allow the selection of the transformed cells. There are two classes of selectable markers: metabolic and antibiotic selectable markers (20). In case of biologics production, some cell lines are preferred to utilize the metabolic selectable markers (e.g.: CHO cells). Two metabolic markers that are widely used for gene amplification are dihydrofolate reductase (DHFR) - methotrexate (MTX) and glutamine synthetase (GS) - methionine sulfoximine (MSX) systems. Both systems can help in generating cells with higher expression level through the increase of transgene copy number (21,22).

1.2.1 Mammalian cells

Different biological hosts, such as bacterial, yeast, and mammalian cells, can be used for recombinant proteins’ production (19). Nevertheless, mammalian cell lines are the preferred option as they possess folding and post-translational machineries similar to the ones present in human cells. This helps to produce proteins with a correct tertiary and quaternary structure, which at same time are less immunogenic (23). Some examples for mammalian expression hosts used in the biopharma are: NS0 murine myeloma cells, PER.C6® human cells, human embryonic kidney (HEK) cells and Chinese hamster ovary (CHO) cells (19). The selection of an expression host is usually determined by its ability to deliver high productivity rate with desired quality, the type of desired biologics to be produced and the preference of individual companies (20).

Chinese hamster ovary cells (CHO) are the number one choice for the host cell with around eighty-six percent of all biologics being produced by them. They are of epithelial origin. Their wide usage is due to several reasons: their ability to grow well in suspension to high cell densities and being relatively robust, hence making such production processes relatively easy to scale-up. At the same time these cells have reasonably high specific productivity, resulting in overall high product yields (24).
1.2.2 Chromosomal integration

Chromosomal integration is a powerful approach to express a gene of interest (GOI) in a host cell, by overcoming the transient expression problems (25). The mechanism of this method relies on the direct integration of any vector that carries the construct after uptake through cell membrane by the host cell. To make such an integration successful, the recipient cell must be in a state of competence for uptake of a foreign DNA, which can either be naturally occurring or induced artificially (26,27).

1.2.2.1 Random integration of foreign DNA constructs

Although instrumentation and modern techniques for recombinant proteins production have evolved significantly in the past years, Random integration (RI) remains one of the principal approaches for integrating a foreign DNA into a host cell under academic and industrial settings (27). It relies on using a recombinant DNA (rDNA), which encodes for POI and a selection marker, and either chemical (e.g. calcium phosphate) or physical (electroporation) transfection. The RI of rDNA in variable sites could lead to a genomic instability and unpredictable behavior of a host cell. In addition, RI is a quite time, labor and cost consuming approach, as it requires more extensive screening procedure to get the most productive clones from a pool of transfected cells. (28).

1.2.2.2 Site-directed integration

In contrast to RI, the site-directed integration (SDI) approach relies on using site-specific recombinases that can induce DNA breakage in a specific region in the genome to integrate and recombine the GOI within the genome (29). There are two evolutionary distinct site-specific recombination mechanisms: serine recombinase, such as ΦC31 integrase; and tyrosine recombinase, such as Cre recombinase (30). Moreover, to increase the number of sites compliant for targeted integration, a combination between catalytic domain of some serine recombinases and some programmable DNA-binding proteins, such as zinc fingers and nuclease de-activated Cas9 (dCas9), was established (31). A reporter gene, such as for enhanced green fluorescent protein (eGFP), is usually introduced into a construct to confirm that the integration process was done properly (32). In comparison with RI, SDI does not require a long clone selection step and provides controllability of the integration events with pre-knowledge of the integration site. Despite several advantages that SI approach harness in comparison to RI approach, it has also a major weakness: the efficiency of SDI can vary depending on the biological host and the specific integration site. Sometimes achieving high levels of integration is difficult and the process may require multiple attempts to achieve the desired result. (33.34).
1.3 Effect of metabolic pathways on mammalian cells and biologics production

For cells to grow and produce the POI, they must have a source of energy. In addition, that source of energy is generated through metabolizing different nutrients through metabolic pathways such as Glycolysis and Krebs cycle. In case of glycolysis (Error! Reference source not found., in cyan), glucose (Gluc) is taken up by the cells to generate ATP which is the unit that gives energy to the cells for biosynthesis. Also, the absorbed glucose could be supplied to the pentose phosphate pathway (PPT) (Error! Reference source not found., in blue) which contributes to redox homeostasis and biosynthesis. The Krebs/tricarboxylic acid (TCA) cycle (Error! Reference source not found., in green) is an alternative approach for cells to get energy, during which cells use the accumulated lactate (Lac) produced and convert it to pyruvate through lactate dehydrogenase enzyme to start producing energy (35). Other metabolic trends are briefly summarized in (Error! Reference source not found.).

Any cell culture that is grown undergoes four different growth phases: lag, exponential, stationary, and death phase. Lag phase is characterized as the first growth phase of the cells, where metabolic activity is present, but growth activity is not active yet. The exponential phase is considered as the most active phase for any cells that are growing, where the viable cell density (VCD) and metabolic activity increase noticeably in the cell culture (36). Furthermore, during such phase cells are known to be very communicative and have higher capacity for nutrient uptake. In case of stationary phase, cells stop growing and finally, at the death phase, cells start to die due to either depletion of nutrients necessary to survive or due to the accumulation of secondary metabolites or waste products which are toxic (37).

The consumption and production of amino acids has been studied in several cell lines (e.g.: CHO cells) during the exponential phase. Several studies have reported a consistent percentage of the contribution of amino acids to TCA cycle replenishment, with glutamine (Gln) as the main carbon source available to the cells through culture media. Glutamine was identified as a contributor with about 40% of the total carbon supply of the TCA cycle (Error! Reference source not found.). Once glutamine is hydrolyzed to glutamate (Glu), it is further converted to α-ketoglutarate which is fed to TCA cycle (38). Cells during metabolic fluxes produce by-products/secondary metabolites such as lactate and ammonium (NH4+), which when accumulated during long term culture could be toxic to the cells or even inhibit the growth of the cells (39).

Metabolic switch is a mechanism where cells switch from one pathway to another to get enough energy for their biosynthesis (39). Normally, during the exponential growth phase during glycolysis, glucose gets oxidized and as a result pyruvate is formed which then is converted to lactate. Several studies have reported that lactate is one of the drivers for the cells to die or stop growing, as lactate accumulation induces growth
inhibition and exhausts the cell machinery (37,38). Furthermore, once cells are depleted from glucose, they tend to switch to consuming lactate and get energy through the TCA cycle instead (35).

Figure 3. Different metabolic pathways for cell biosynthesis. Cyan: glycolysis, blue: pentose phosphate pathway, green: TCA/Krebs cycle.

Adapted from: (Coulet et al, 2022)

This thesis is a part of the process development project in the protein and viral production, research, and development (R&D) section at Cytiva. The main aim for this project was to establish a clone selection platform and to identify the best producing clone from a specific cell line and its clones from the metabolic point of view. These cell lines were established through SDI.
2. Objectives

The objectives of this project were to:

- Identify the most adequate feed rate for future clone selection platform based on this cell line(s) and fed-batch regime.

3. Experimental design

The figure below illustrates the experimental design of the project (Figure 4), where three cell line clones from a Chinese hamster ovary (CHO) cell line were tested in the project (see “Input”). For each cell line, three rounds of passaging (seed trains) were applied before running batch or fed-batch culture mode (see “Process”). The cell line CHO-SDI-Z was engineered as the original clone and the first cell line, while CHO-SDI-X and CHO-SDI-Y were derived from the CHO-SDI-Z. CHO-SDI-Z was originally engineered by inserting a cassette which expresses the mAb bound to another POI. While CHO-SDI-Y was established through removing the aforementioned POI at which the insert only carries the mAb. The CHO-SDI-X cell line was engineered by removing both the mAb and the POI, so it contained an empty insert. Finally, cell specific feed rate strategy was implemented as the main strategy for cell feeding during fed-batch study, at which each cell line clone was fed accordingly when they reached the middle of the exponential growth phase (Figure 4, see output).

<table>
<thead>
<tr>
<th>Input</th>
<th>Process</th>
<th>Output</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-SDI-X</td>
<td>Seed trains</td>
<td>Cell recovery/stabilize doubling time (PDT)</td>
</tr>
<tr>
<td>CHO-SDI-Y</td>
<td>Batch culture</td>
<td>Scale up cell culture volume</td>
</tr>
<tr>
<td>CHO-SDI-Z</td>
<td>Fed-batch culture</td>
<td>Cell kinetics <em>(Viable cell density, viability)</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metabolic profile <em>(Gluc, Lac, Gln, Glu, NH4+)</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Feeding start day for cell specific feed strategy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell kinetics <em>(Viable cell density, Viability)</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metabolic profile <em>(Gluc, Lac, Gln, Glu, NH4+, IgG)</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell specific feed rates <em>(0.5, 0.75, 1.125pl/cell/day)</em></td>
</tr>
</tbody>
</table>

Figure 4. Experimental design of the study.
4. Materials and Methods

The Chinese hamster ovary cell lines (CHO) and the engineered clones that were used throughout this project are listed in Table 1.

Table 1. List of cell lines.

<table>
<thead>
<tr>
<th>Cell line origin</th>
<th>Cell line name</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO cells (CHO-SDI-Z)</td>
<td>CHO-SDI-X</td>
</tr>
<tr>
<td>CHO cells (CHO-SDI-Z)</td>
<td>CHO-SDI-Y</td>
</tr>
<tr>
<td>CHO cells</td>
<td>CHO-SDI-Z</td>
</tr>
</tbody>
</table>

4.1. Seed Train

Before every passage, batch and fed-batch culture, Actipro media was supplemented with 6 mM glutamine (Gln) and conditioned in shake incubator at 37°C/ 105rpm/ 5% CO2/ 80% humidity for minimum of 45 min (see Table 2 for used media and solutions; Table 3 for used equipment).

Table 2. List of media and solutions.

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actipro media</td>
<td>Actipro:22.36 g/L</td>
</tr>
<tr>
<td>Glutamine</td>
<td>6 mM</td>
</tr>
<tr>
<td>Cell boost 7a</td>
<td>7a: 181.04 g/L</td>
</tr>
<tr>
<td>Cell boost 7b</td>
<td>7b: 94.6 g/L</td>
</tr>
</tbody>
</table>

Table 3. List of equipment.

<table>
<thead>
<tr>
<th>Item</th>
<th>Manufacturer</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell counter</td>
<td>Beckman</td>
<td>ViCell XR cell counter</td>
</tr>
<tr>
<td>Laminar flow hood</td>
<td>Savvy</td>
<td>SAVVY SL Class II</td>
</tr>
<tr>
<td>Metabolic analyser</td>
<td>Roche</td>
<td>CedeX HT bio</td>
</tr>
<tr>
<td>Osmometer</td>
<td>Advanced instrument</td>
<td>OsmoTECH PRO Multi-Sample</td>
</tr>
<tr>
<td>Shake incubator (50 mm throw)</td>
<td>Kuhner</td>
<td>Climo-shaker ISF1-XL</td>
</tr>
</tbody>
</table>

4.2. Thawing and passaging:

Thawing:

One cryovial at a time was thawed in a shake incubator at 37°C for 3-5min. Cells were washed with 10mL of previously preconditioned ActiPro + 6mM Gln media/ 1ml of cell from the cryovial, followed by centrifugation at 300g for 5min at room temperature (RT) and subsequent removal of supernatant, except
for the final 3mL, which were used to resuspend the cell pellets. Finally, viable cell densities (VCD), viability and cell size were measured using Vi-Cell XR cell counter.

**Subculturing/Passage**

Target initial and end viable cell densities (VCD) for each seed-train step were $0.5 \times 10^6$ (±0.1), and 4±1 MC/mL, respectively. For the split ratios <5, cells were washed by centrifugation at 300g at room temperature (RT) for 5min, followed by resuspension in fresh and pre-conditioned media. The cell culture to flask volume ratios were maintained constant at 1:5. Incubation conditions were 37°C/ 7.5% CO₂/ 80% humidity/ 105rpm/ 72±4h. Samples were collected, and VCD and viability were measured.

### 4.3. Batch and fed-batch cultures

For batch experiments, each cell line clone was cultured in triplicates, while for fed-batch in duplicates per feed condition. If needed (VCD≥10 MC/mL), 1xPBS solution was used for dilution 1:1 (dilution factor 2).

**Inoculation and sampling:**

For each cell line clone (CHO-SDI-X, CHO-SDI-Y, CHO-SDI-Z), a master shake flask was prepared, and cells were split from the previous passage to a VCD of $0.5 \pm 0.1$ MC/mL. The master shake flasks were left at 37°C/ 7.5% CO₂/ 80% humidity/ 105rpm/ 5min prior sampling for VCD, viability and metabolites’ measurements. Each master shake flask was split into 1L shake flasks with 205(±2) mL of cell culture (the ratio of the cell culture volume to the volume of the shake flask is 1:5). 25mL of stock media supplemented with glutamine was passed to 125ml flask for contamination control flasks (negative control). On a daily basis, 1.7mL of samples were taken from each cell flask for measurements of VCD, viability, osmolarity and metabolites (Lactate (Lac), Glutamine (Gln), Glutamate (Glu), Ammonia (NH₄⁺), Glucose (Gluc), and IgG titre). For fed-batch experiments sampling volume was adjusted to maintain constant cell culture volumes of 200mL.

**Fed-batch:**

The onset of the fed-batch mode corresponded with the middle of exponential phase of each cell line for a given basal media.

Three different feed rates were tested for each cell line clone as a cell boost: 0.5, 0.75, and 1.125 pL/cell/day of both cell boost 7a and 7b is one tenth the concentration of cell boost 7a (CB7a and CB7b), respectively. In addition, Gluc was introduced to the cells as additional feed and maintained at concentration up to 4g/L during Lac production phase.
4.4. Equations and formulas

The following equations and formulas were used:

\[ \text{PDT} = \mu \times 24 \text{ (hours)} \] where:
- PDT= population doubling time (hours)
- \( \mu = \) growth rate (1/day)

\[ N = N_0 e^{\mu T} \] where:
- N= cell number (MC/mL)
- N0= Starting cell number
- \( \mu = \) growth rate (1/day)
- T= time (hours)

\[ \text{VCC} = \text{VCD} \times \text{Vshake flask} / 10^3 \text{, where:} \]
- VCC= viable cell count (x10^9 cell)
- VCD= viable cell density (MC/mL)
- Vshake flask= Volume in the shake flask (mL)

\[ \text{IVCC} = \text{IVCC}_t + \text{IVCC}_{t+1}, \text{ where:IVCC}_t = (\text{VCC}_{t+1} + \text{VCC}_t) / 2 * (t-t_{t-1}) \]
- IVCC= Integral viable cell count (10^9 cells/day)
- VCC= viable cell count (x10^9 cell)
- t= time (Days)

\[ qP = \frac{\text{slope } (\Delta N / \text{IVCC}_c)}{10^3}, \text{ where:} \]
- qP= cell specific metabolic rates (pg/(cell/day)) or (mmol/(cell/day))
- \( \Delta N = \) Change in the amount of the metabolite (g/mmol)
- \( \text{IVCC}_c = \) integral cell count (10^9 cells * day)
5. Results

5.1 Seed Trains

After thawing, cells were passaged (seed trains) at least three times prior the batch or the fed-batch culture. During the seed trains, population doubling time (PDT) was measured for each cell line for each passage. PDT reflects how many hours the cell needed to be doubled during each passage/seed train. The graph below (Figure ) shows the measured PDT in hours (h) for each cell line clone after passaging i.e. for passages 1, 2 and 3, respectively. The x-axis (time) reflects the time in culture for the cell lines (11 days), during which the cells were passaged 3 times. The PDT from different passages for CHO-SDI-X was 17.8, 19.08, 19.8 hours respectively. In case of CHO-SDI-Y it was 17.735, 20.09, 18.8 hours respectively, while for the last cell line CHO-SDI-Z PDT was 19.5, 22.2, 21.82 hours respectively. Using T-test, statistical testing showed significance PDT difference with p value= 0.045 between CHO-SDI-Z and CHO-SDI-X. While between CHO-SDI-Z and CHO-SDI-Y slight significance with p value= 0.05. Finally, there was no significant difference between the PDT of CHO-SDI-X and CHO-SDI-Y.

Figure 5. Cell line clone’s population doubling times (PDT) during seed trains. The graph represents the population doubling time for each cell line during each passage during the 11 days. The X-axis represents the culture time in days while y-axis represents the PDT in hours.
5.2 Batch experiments
The batch experiment for CHO-SDI-X (Figure ) cell line clone took around nine days till most of cells died. The starting VCD of the batch experiment for this cell line clone was 0.5±1 MC/mL. The cell line reached the middle of the exponential phase on day three, and the VCD was 5.94 MC/mL (Figure (A)). The cells started to shift from the exponential growth phase on day four and enter the stationary phase (Figure (B)). In addition, this cell line showed a peak VCD of 17.28 MC/mL, while the PDT was 23.1 hours (data not shown). After day nine the culture was stopped with VCD of 11.45 MC/mL and viability 66%. Furthermore, the metabolic profile of the cell line which indicates the consumption and production rate of different metabolites, showed different depletion moments of certain metabolites within the cell line (Figure (C)). Gln depletion from the cells started from day five, while Gluc depletion happened on day six. In addition, after glucose reached critical concentration on day five, it was observed that cells started to consume the accumulated produced lactate, switching from lactate production to lactate consumption (Figure (C)) (supplementary figure.1).
Figure 6. Cell culture kinetic and metabolic profiles of CHO-SDI-X: A) VCD and viability vs time; B) ln(VCC) vs time; C) growth rate and cell specific metabolic rates vs time. The blue and yellow vertical lines mark the middle and the end of the exponential growing phase. While the green and black mark the Gln and Gluc depletion moments, respectively, and the blue dotted line marks the start of Lac consumption.

For CHO-SDI-Y, the culture duration until cell death was also nine days (Error! Reference source not found.). Cells’ initial VCD was 0.5± MC/mL after inoculation. The cell line clone reached the middle of the exponential phase on day three, and the VCD was 4.77 MC/mL (Error! Reference source not found.(A)). In addition, the cells started to shift from exponential phase to stationary phase on day four (Error! Reference source not found.(B)), while the PTD was 20.88 hours (data not shown). Furthermore, the metabolic profile of the cell line clone showed the different depletion moments of certain metabolites, at which Gln depletion happened on day five while Gluc depletion happened on day six. Finally, cells started to switch to consume the accumulated produced lactate on day five (Error! Reference source not found.(C)) (supplementary figure.1).
Figure 7. Cell culture kinetic and metabolic profiles of CHO-SDI-Y: A) VCD and viability vs time; B) ln(VCC) vs time; C) growth rate and cell specific metabolic rates vs time. The blue and yellow vertical lines mark the middle and the end of exponential growing phase. While the green and black mark the Gln and Gluc depletion moments, respectively, and the blue dotted line marks the start of Lac consumption.

In case of CHO-SDI-Z, the culture duration until the cells died took six days. Cells were also inoculated at 0.5± MC/mL. Although this cell line reached the middle of the exponential growth phase and left the exponential phase at the same days as the previous two cell lines (day 3 and 4, respectively), it showed higher VCD in comparison to the other two mentioned cell line clones (Error! Reference source not found.). The VCD was 8.44± MC/mL in the middle of exponential phase (Error! Reference source not found. (A)). In addition, the PTD was 16.75 hours (data not shown). Finally, CHO-SDI-Z showed earlier depletion moment in comparison with the other two cell line clones, where Gln depleted on day four and Gluc on day five. Cells started to switch to lactate consumption on day four (Error! Reference source not found. (C)) (supplementary figure.1).

Figure 8. Cell culture kinetic and metabolic profiles of CHO-SDI-Z: A) VCD and viability vs time; B) ln(VCC) vs time; C) growth rate and cell specific metabolic rates vs time. The blue and yellow vertical lines mark the middle and the end of exponential growing phase.
The table below represents a summary of the batch experiment. Which shows the VCD of each cell line in the middle of the exponential phase, PDT, and the highest VCD and viability that was reached for each cell line. Finally, the different depletion day points for different metabolites (Gluc, Lac, Gln, Glu).

Table 4. Summary table for the batch study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Doubling time (Exp.) (h)</th>
<th>VCD (mid Exp) (x10^9 cell/mL)</th>
<th>VCD (END Exp) (x10^9 cell/mL)</th>
<th>Peak VCD (x10^9 cell/mL)</th>
<th>Peak viability (%)</th>
<th>Gluc Depletion</th>
<th>Lac Shift</th>
<th>Gln depletion</th>
<th>Glu consumption</th>
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<tr>
<td>CHO-SDI-X</td>
<td>23.1</td>
<td>5.94</td>
<td>11.88</td>
<td>17.28</td>
<td>98.05</td>
<td>Day 6</td>
<td>Day 5</td>
<td>Day 5</td>
<td>Day 4</td>
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<tr>
<td>CHO-SDI-Y</td>
<td>20.88</td>
<td>4.77</td>
<td>9.53</td>
<td>17.28</td>
<td>97.23</td>
<td>Day 6</td>
<td>Day 5</td>
<td>Day 5</td>
<td>Day 4</td>
</tr>
<tr>
<td>CHO-SDI-Z</td>
<td>16.75</td>
<td>8.44</td>
<td>16.88</td>
<td>19.10</td>
<td>86.65</td>
<td>Day 5</td>
<td>Day 4</td>
<td>Day 4</td>
<td>Day 3</td>
</tr>
</tbody>
</table>

5.3 Fed-batch experiments

Fed-batch experiments were performed for all three abovementioned cell line clones: CHO-SDI-X, CHO-SDI-Y and CHO-SDI-Z.

The graphs below (Figure.9) represent the cell kinetics and metabolic profile of the cell line CHO-SDI-X during the fed-batch experiment. Initially, the starting VCD for cells was 0.5±(0.1) MC/mL, while the culture duration was twelve days in total. Cells were fed with glucose, cell boost 7a and 7b from day three according to the cells’ glucose levels, VCD and viability (Figure.9 (A)). Cells that were fed at the highest feed rate (1.125 pL/cell/day) showed higher VCD peaks but also these cells had the most decline rate and died suddenly on the last day (Figure.9 (B)). In addition, cells fed at feed rates 0.5 and 0.75 pL/cell/day reached their peak VCD on day six, while for the feed 1.125 pL/cell/day the peak VCD was on day seven (Figure.9 (A)). For the cell line metabolic profile, depletion/reaching critical concentration of glutamine was observed on day five. While glutamate depletion was observed on day six for the two lower feed conditions (0.5 and 0.75 pL/cell/day) but not for the highest feed condition (1.125 pL/cell/day), where it happened on day seven (Supplementary figure.2 (D)). Furthermore, cells with lowest feed rate showed consumption of lactate instead of its production from day eleven (Figure.9 (C)), (supplementary figure.2 (B)). Finally, at the end of the experiment on day thirteen, the viability for the cells that were treated with...
either 0.5 or 0.75 pL/cell/day was around 80%, while cells treated with the highest feed rate (1.125 pL/cell/day) were at viability of 15% (Figure 9 (A)).

![Figure 9](image_url)

Figure 9 Cell culture kinetics and metabolic profiles of CHO-SDI-X in a fed-batch mode: A) VCD and viability vs time; B) ln(VCD) vs time; C) growth rate and cell specific metabolic rates vs time. The green color represents cells treated with 0.5 pL/cell/day feed rate, purple color represents cells that were treated with 0.75 pL/cell/day feed rate and blue color is for the cells with 1.125 pL/cell/day. In addition, the red vertical line marks the feeding start day. While the yellow, dark yellow and grey ones mark the Gln, Lac, and Glu, NH3+ depletion moments, respectively.

For the cell line CHO-SDI-Y (Figure), culture duration was also twelve days in total and the starting VCD for cells were 0.5±(0.1) MC/mL. Cells were fed with glucose, cell boost 7a and 7b from day three according to the cells’ glucose levels, VCD and viability. Similar to the previous cell line clone, cells treated with the two lower feed rates (0.5 and 0.75 pL/cell/day) showed highest VCD on day six; while cells which were fed with the highest feed rate (1.125 pL/cell/day) showed highest VCD peaks on day seven, but also showed the most decline rate and died suddenly on the last day (Figure (A, B)). For the cell line metabolic profile, depletion/reaching critical concentration of glutamine was observed on day five, while glutamate depletion was observed on day six for the two lowest feed conditions (0.5 and 0.75 pL/cell/day) (Supplementary figure.3 (D)). Furthermore, cells which were treated with lowest feed rate were found to shift to lactate...
consumption instead of lactate production from day eleven (Figure (C)), (supplementary figure.3 (B)). Finally, at the end of the experiment on day thirteen, the viability for the cells that were treated with (0.5 or 0.75 pL/cell/day) was around 70%, while cells that were treated with the highest feed rate (1.125 pL/cell/day) were at viability of around 12% (Figure.10 (A)).

Figure 10. Cell culture kinetics and metabolic profiles of CHO-SDI-Y in a fed-batch mode: A) VCD and viability vs time; B) ln(VCC) vs time; C) growth rate and cell specific metabolic rates vs time. The green color represents cells treated with 0.5 pL/cell/day feed rate, purple color represents cells that were treated with 0.75 pL/cell/day feed rate and blue color is for the cells with 1.125 pL/cell/day. In addition, the red vertical line marks the feeding start day. While the yellow, dark yellow, and grey ones mark the Gln, lactate consumption and Glu depletion moments, respectively.

In case of CHO-SDI-Z cell line clone (Figure ), the duration of the culture was twelve days and the starting VCD of the cells were 0.5±(0.1) MC/mL. Feeding started accordingly from day three just like the previous two cell lines. For CHO-SDI-Z, the tested different feed rates showed different VCD peaks on day six (Figure (A, B)). In addition, it was observed that most of the cells in each shake flasks from this cell line died (all cells that were fed with 0.75, 1.125 pL/cell/day feed rates). Except the cells that was fed with the lowest feed rate (0.5 pL/cell/day) they were still alive with viability of 80% (Figure (A, B)). For the cell
metabolic profile (Figure (C)), it was observed that this cell line also has different/earlier depletion moments in comparison to the previous cell lines: Gln depleted on day five, whereas Glu depletion occurred on day five for cells that were fed at 0.5 or 0.75 pL/cell/day feed condition (Supplementary figure 4 (D)). Finally, at the end of the experiment (day 12), the cells’ viability dropped to around 40% for cells that were treated with 0.5 pL/cell/day and around 8% for the cells that were treated with the higher feed rate conditions (Figure (A)).

The table below represents a summary of the fed-batch experiment. Which shows the highest VCD and viability that was reached for each cell line with each tested feed rate. And the different depletion day points

![Cell culture kinetics and metabolic profiles of CHO-SDI-Z in a fed-batch mode](image)

Figure 4. Cell culture kinetics and metabolic profiles of CHO-SDI-Z in a fed-batch mode: A) VCD and viability vs time; B) ln(VCC) vs time; C) growth rate and cell specific metabolic rates vs time. The green color represents cells treated with 0.5 pL/cell/day feed rate, purple color represents cells that were treated with 0.75 pL/cell/day feed rate and blue color is for the cells with 1.125 pL/cell/day. In addition, the red vertical line marks the feeding start day. While the yellow and grey ones mark the Gln and Glu depletion moments, respectively.
for different metabolites (Lac, Gln, Glu). The “--” refers to no switch/depletion moment happened in the specific condition/cell line.

In addition, osmolality levels during the three last days of fed-batch experiments were measured for all three cell-line clones (Figure). It was observed that the osmolality of the cell cultures gradually rose to high levels during the last three days. Cells that were fed with highest fed rate (1.125 pL/cell/day) showed elevated levels of osmolality which correlates with the sudden drop of the viability and the sudden death of the cells.

Table 6. Summary table for Fed-batch experiment

<table>
<thead>
<tr>
<th>Name</th>
<th>Peak VCD (x10^6 cell/mL)</th>
<th>Peak Viability (%)</th>
<th>Glu Depletion</th>
<th>Glu depletion</th>
<th>Lactate switch</th>
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<tr>
<td>CHO-SDI-X (0.5pL/cell/day)</td>
<td>24.35</td>
<td>99.5</td>
<td>DS</td>
<td>D6</td>
<td>D11</td>
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<tr>
<td>CHO-SDI-X (0.75pL/cell/day)</td>
<td>26.15</td>
<td>98.45</td>
<td>DS</td>
<td>D6</td>
<td>--</td>
</tr>
<tr>
<td>CHO-SDI-X (1.125pL/cell/day)</td>
<td>27.40</td>
<td>99.55</td>
<td>DS</td>
<td>D6</td>
<td>--</td>
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<tr>
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<td>26.45</td>
<td>98.5</td>
<td>DS</td>
<td>D6</td>
<td>D11</td>
</tr>
<tr>
<td>CHO-SDI-Y (0.75pL/cell/day)</td>
<td>28.75</td>
<td>98.9</td>
<td>DS</td>
<td>D6</td>
<td>--</td>
</tr>
<tr>
<td>CHO-SDI-Y (1.125pL/cell/day)</td>
<td>29.7</td>
<td>95.25</td>
<td>DS</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>CHO-SDI-Z (0.5pL/cell/day)</td>
<td>27.7</td>
<td>97.07</td>
<td>DS</td>
<td>D5</td>
<td>--</td>
</tr>
<tr>
<td>CHO-SDI-Z (0.75pL/cell/day)</td>
<td>30.15</td>
<td>97.9</td>
<td>DS</td>
<td>D5</td>
<td>--</td>
</tr>
<tr>
<td>CHO-SDI-Z (1.125pL/cell/day)</td>
<td>32.4</td>
<td>98.8</td>
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<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
6. Discussion

This thesis project was an important step in establishing a successful clone selection platform to select for the best cell clones producing proteins of interest. The hypothesis of the project was that the three cell-line clones were established by integration of the insert into the same locus by SDI approach. Furthermore, the
location of the insertion in the genome was confirmed in previous studies at the company. Thus, by avoiding any random insertion or genomic rearrangement that could possibly make the cell line clones unstable, the current setup has a big advantage of using the SDI over RI (37,45).

Seed trains are crucial and important strategies in a process of clone selection from any set of cell line clones that come from same origin. The idea of seed trains, which is passaging the cells several times (three times ideally), is to scale up the culture volume, especially for the cells grown in suspension, and to generate enough cells prior to inoculation into a bioreactor, as an example. Also, seed trains are necessary to give the cells enough time to recover from the stock thawing process before starting any batch or fed-batch experiments. Another important reason for the seed train step, is to maintain the doubling time of each cell line in order to maintain their similar growth patterns and kinetics. After applying the seed trains in the current study, we observed that the cell line CHO-SDI-Z presented with a different doubling time in comparison to the other cell line clones, which also reflected on the cell line growth rate and VCD during passages. Based on our preliminary findings, we suggest that the CHO-SDI-Z cell line may present with initial cell kinetics different from the other two cell line clones. Regarding statistical testing, using T-test was to compare each cell with the other independently, since T-test analyse two groups at a time. An alternative approach for statistical testing is to use one-way ANOVA test to compare the 3 groups in one test and compare their means which would be more efficient and less time consuming, since ANOVA can compare three groups at one time.

For identification of the exponential growth phase of each cell line clone, indicating the ideal starting point for feed experiments, we performed the batch experiments. During the exponential phase of cell growth, the cells are known to have higher uptake for the nutrients and are more responsive to feeding, which boosts the growth of the cells and results in higher count in VCD (39). During batch culture experiment, we observed that the CHO-SDI-Z cells have different cell kinetic behaviour than both CHO-SDI-X and CHO-SDI-Y. More specifically, we observed that CHO-SDI-Z showed shorter doubling time and reached higher VCDs at the middle of the exponential phase and higher peak VCD. Although the middle of the exponential phase for the three cell lines was identified on day three, the cell line CHO-SDI-Z showed elevated VCD at such point in comparison to the other cell lines. Such observations indicate that CHO-SDI-Z may present with different cell kinetics from the other two cell line clones.

For the final goal of characterization of the metabolic profile of the three CHO cell line clones, we measured the daily concentration of certain metabolites (Gluc, Lac, Glu, Gln, NH4+) and subsequently determined
their specific consumption and production rates. We observed that CHO-SDI-X and CHO-SDI-Y cells exit the exponential growth phase after the depletion of Gluc from day six, whereas CHO-SDI-Z from day five, accompanied with the growth rate of the cells starting to decrease. After depletion of Gln and Gluc, the CHO-SDI-X and CHO-SDI-Y cells started to lose their viability and die, whereas for CHO-SDI-Z cells it was unclear if any depletion moment directly affected the cells’ viability. In addition, we could identify a metabolic switch to Glu-Gln cycle (consuming Glu and NH4+) once glutamine was depleted from the cells. Glutamine production is necessary for cells’ division and biosynthesis processes. Such observations were expected, as the cells use both metabolites (Gln) as the main carbon sources to generate energy for biosynthesis. In addition, CHO-SDI-Z showed different depletion patterns from the other two cell line clones, which was expected based on the different cell kinetic identified. Since CHO-SDI-Z cell line clone showed lower doubling time and higher VCD than the other two cell line clones, it most probably resulted in the cells consuming glucose and glutamine earlier, leading to the cells switching to lactate and glutamate consumption and reaching the metabolic switch earlier than the other two cell lines.

Although the three cell-line clones had been engineered through SDI approach, it was previously confirmed by sequencing that the integration occurred at the same, correct location. The CHO-SDI-Z clone was historically the first one generated, while the other two cell lines were further derived from it. A possible explanation for differences in cell kinetic and metabolic profiles between CHO-SDI-Z and the other two cell lines could be that during the process of removing the recombinant protein attached to the mAb for CHO-SDI-Y cell line and removing both the recombinant protein molecule and mAb in case of CHO-SDI-X, an additional disruption in the genome might have occurred. Specifically, if the regulatory elements, such as promoters or enhancers, were involved it could have affected the cells’ metabolic machinery and behaviour. According to several studies it is important that the regulatory elements are located at a greater distance from the gene they control, as alteration or disruption on these elements would affect the gene expression reflecting on the cell machinery (43, 44). Another possible explanation is the nature of the recombinant protein had influence on cell machinery. Given that the three cell lines carried different constructs at the end, these modifications may have the potential to alter their genomic landscapes. Such alterations could subsequently influence cellular kinetics and the metabolic profile. To validate these hypotheses, further investigation is needed. Future studies should employ a new cohort of another set of cell line clones engineered with the identical conditions and expressing the same recombinant protein. These clones would then be comparatively evaluated for their cell kinetics and metabolic profiles.
Optimal conditions for production of a protein of interest by cloned mammalian cell lines is a very important step during a process of recombinant protein production. This process is performed via fed-batch experiments that identify how much the cells could expand, produce POI, when they’re fed regularly with the needed nutrients. Furthermore, to identify how much the cells can withstand before they get overfed or underfed when they are supplemented with different feed rate of the necessary nutrients (cell boost). According to study by Romanova et al (2022), CHO cells achieve maximum productivity with higher peaks of cell density and higher product titer of the recombinant protein of interest when they are cultivated in fed-batch mode. In addition, feeding them can promote higher production and limit the nutrient depletion problem (45).

According to Horvat et al (2020), it is important to design an adequate feed strategy with proper concentrations to produce high-yield and high-quality of recombinant proteins (46). Furthermore, adjusting the feeding strategy to be cell specific according to the cell concentration rather than a fixed ratio, hold some advantages. Such as, enhancing cells productivity by tailoring the feed strategy according to the cell’s dynamics and requirements on a daily basis, which maintain the optimal environment for the cells and improve productivity. Another advantage is that it helps to reduce variability and help to have more precise and controlled environment for the cells to grow in (47, 48).

Previously, an optimal feed rate for another set of CHO cell line clones has been identified as 0.75 pL/cell/day (unpublished confidential data). However, we decided to test two additional feed rates - 0.5 and 1.125 pL/cell/day in order to verify the most adequate feed rate and to set a threshold for feeding rates for this group of cells. We observed that cells were underfed at the 0.5 pL/cell/day rate, mirrored in lower peak VCDs and slower growth in comparison to cells treated with other feed rates. Cells fed at 1.125 pL/cell/day rate were identified as overfed on the last day(s) of culture. Since the cell cultures exhibited a dramatic drop in the VCD and viability, as well as a simultaneous drastically higher osmolality, which is an indicator of over feeding. Therefore, our results identified 0.75 pL/cell/day feed rate as the most adequate, among the tested feed rates, matching with the results of a previous experiment, although performed on a different set of CHO-cell lines. As a recommendation for future studies, other conditions could be tested with different feed rates. As long as, they are less than 1.125 pL/cell/day and more than 0.5 pL/cell/day,

A follow-up evaluation of the cell kinetics and metabolic profile during the fed-batch experiments was the ultimate goal of our study. We observed that cells during fed-batch mode survived longer (12 days) in comparison to batch culture (8 days), which was expected since cells were fed with boosting agents starting
from the middle of their exponential growth phase. In addition, beside supplementing the cells with cell boost, glucose was also introduced as supplementation to the cells to maintain the glucose concentration at 4g/L. As, the main reason for that, is to hold the cells in their exponential growth phase as long as possible by continuous feeding. It is known that lactate production usually increases during the exponential growth phase. At which cell tends to consume glucose and produce lactate. While when cells start to enter the stationary phase, they tend to switch to lactate consumption instead of glucose. This process of lactate shifting is still not completely understood yet (39).

Similarly to the batch experiments, CHO-SDI-Z showed different cell kinetics and metabolic profile in comparison to the other two cell line clones, illustrated by higher VCD peaks and different/earlier depletion timepoints when it comes to certain metabolites (Glu). Although, the Gluc levels were maintained constant during the experiment (4g/L), the Gln and Glu depletion events showed some association with the cell growth rate - with cell growth rate starting to drop after Gln and Glu were depleted from the cells. Finally, we noticed that each cell line clone with each cell feed rate started to reach their peak VCD once Gln and Glu started to deplete or reach very critical concentration. According to several studies, glutamine is crucial as a nutrient for cells to proliferate and depletion of such an amino acid would affect cell growth and proliferation drastically (49, 50). In conclusion, our findings from the fed-batch experiment match with our batch experiment, opposing to the project original hypothesis and confirming that the cell line clones have different phenotypes and metabolic profiles. Especially, CHO-SDI-Z clone seems to present with different behaviour than the other two cell lines.

Another interesting finding from the fed-batch experiment with CHO-SDI-X and CHO-SDI-Y clones showed that the cells fed at the lowest rate (0.5 pL/cell/day) switched towards lactate consumption on day eleven. This could potentially be a result of underfeeding and/or induced by pH changes, as according to several studies, the process of pH fluctuations has been shown to be capable of inducing the lactate metabolic shift (51, 52, 53).

Lastly, we observed that there is a big difference in the titer and specific productivity of the biologic of interest (IgG) across the two cells lines that produce recombinant protein (CHO_SDI-Y and CHO_SDI-Z). The main reason for such differences is most probably that the mAb that CHO-SDI-Z expresses is bound to another recombinant protein. Since the IgG titer was measured by CedeX HT bio instrument, it detects the antibody through the interaction of IgG with protein A. Protein A is known by its high affinity to Fc region of immunoglobulins and such interaction can be used as detection methods for various
immunoglobulins. Looking at our data, we hypothesize that, since the POI expressed in CHO-SDI-Z consists of two subunits, one being the immunoglobulin (mAb) and the other a fused protein, the competitive binding between protein A and both subunits, caused by steric hindrance, may have occurred. Such steric hindrance may have led to the low detection levels of IgG in CHO-SDI-Z, resulting in a false measured IgG titer. An optimal solution for the problem but more expensive, is to use biacore system to measure the titer. Biacore has different technology to measure concentrations, based on surface plasmon resonance. Which, is more specific approach to measure the titer, also such system offers real-time monitoring to measure protein-protein interaction. (54).

This project is part of process development initiative to design an optimized platform that helps in the adequate selection of the best producing cell line clone in a larger scale. Another aspect of the project is to test each cell maximum capacity in terms of expansion and productivity by exploring the cell kinetics and metabolic profile for each cell line clone. Further follow up studies should take place in order to optimize the platform for larger scale to scale from large scale shake flask culture to bioreactors. Another aspect that needs to be tested is to investigate more the most adequate feed rate by checking different feed rates that range from 0.6 to 1 pL/cell/day. Finally, biacore can be tested as an optimal alternative for measuring the IgG titer.

7. Conclusions
In summary, the following goals of the project have been reached:

1. The starting point of fed-batch regime was determined for each tested cell line.
2. The driving factors for the end of the exponential phase and end of stationary phase have been identified for some of the cell cultures.
3. The optimal feed rate was identified.

8. Acknowledgements
I would like to thank my supervisors Srdja and Thomas for their dedicated effort and constant help toward me. I also would like to thank everyone in the large-scale team (Malin, Sanna, Henrik) for helping me out and providing support during my time in the company. Finally, would like to thank Bahareh, CLD team and the section manager Henrik Johannesson.
9. References


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Supplementary material

Supplementary table 1. List of consumables

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<th>Item</th>
<th>Manufacturer</th>
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<td>Shaker flasks (125, 250, 500, 1000 mL)</td>
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<td>ViCell Reagent kit</td>
<td>Beckman</td>
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Supplementary table 2. List of chemicals

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<td>Glutamine</td>
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<td>Cytiva</td>
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<td>Merck/Sigma</td>
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Supplementary table 3. List of equipment

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<td>pipette 1mL</td>
<td>Metech</td>
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<tr>
<td>pipette 200µL</td>
<td>Metech</td>
</tr>
<tr>
<td>Scale</td>
<td>Mettler-Toledo</td>
</tr>
</tbody>
</table>

Supplementary results

The graphs below (Supplementary figure 1.) represents the concentration of different metabolites, during batch culture experiment for the 3 cell lines (Gluc, Lac, Gln, Glu, NH4+).
Supplementary figure 1 (A, B, C, D, E): the figures above represents the concentration of different metabolites for the cell lines (Gluc, Lac, Gln, Glu, NH4+), during batch culture experiment.
The graphs below (Supplementary figure 2, 3, and 4.) represent the concentration of different metabolites, during fed-batch culture experiment for the 3 cell lines (Gluc, Lac, Gln, Glu, NH4+).

Supplementary figure 2 (A, B, C, D, E): the figures above represent the concentration of different metabolites for CHO-SDI-X in different feed conditions (Gluc, Lac, Gln, Glu, NH4+), during fed-batch culture experiment.
Supplementary figure 3 (A, B, C, D, E, F): the figures above represent the concentration of different metabolites and IgG titer for CHO-SDI-Y in different feed conditions (Gluc, Lac, Gln, Glu, NH4+), during fed-batch culture experiment.
Supplementary figure 4 (A, B, C, D, E, F): the figures above represent the concentration of different metabolites and IgG titer for CHO-SD1-Z in different feed conditions (Gluc, Lac, Gln, Glu, NH4+), during fed-batch culture experiment.