Process Transfer and Optimization of Chinese Hamster Ovary Cell Cultivation for Monoclonal Antibody Production

Von der Naturwissenschaftlichen Fakultät der

Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktorin der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation

von

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[2021]

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Tag der Promotion:	23.02.2021

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

Dr Marie Skłodowska Curie

Abstract

Biopharmaceutical market has been progressively expanding, and moving away from small molecular drugs to biotechnologically produced therapeutics such as recombinant proteins. Genetically modified mammalian cells, such as Chinese Hamster Ovary cells, are being used extensively for the production of these proteins. However, a challenge faced by the biopharmaceutical industry is to attain maximum product within a limited culture volume due to volume constraints in the bioreactor. This is overcome by intensifying the process and understanding the fundamentals of cell growth, which varies across different cell strains.

The main objective of this study was to successfully transfer and scale the process in different types of bioreactors with a working volume ranging from 15 mL to 50 L. Process attributes, namely, viable cell concentration and final titre quantity were used to evaluate the scalability of the process. It was shown that the process was robust and scalable across different types of bioreactors.

The second part of the project was to optimize the cultivation process in terms of testing the process parameters that control cultivation, primarily, the dissolved oxygen (DO) concentration and pH. We identified that reducing the DO to 40% and maintaining the pH at 7.1 not only decreased the requirement of pure oxygen in production scale bioreactors, but also reduced the damage to cultivated cells caused by oxygen driven free radicals.

The next part of process optimization was conducted by varying the concentrations of ingredients in production medium and feed media used for the fed batch process. Concentration of carbon (glucose) and nitrogen (glutamine and glutamate) sources in the production medium were altered and the impact on the viable cell concentration and protein production was studied. The results showed that the production medium can be further improved by altering the initial concentration of glutamine and glucose to range between 0.6 to 1.2 g/L and 6 to 12 g/L, respectively. Glutamate was essentially used for protein production and was supplemented to the culture through the feed medium. Therefore, was not required to be added additionally in the production medium.

In order to optimize the percentage of feed medium, different concentrations of the two feed media (FMA and FMB) were added to the cell culture. It was shown that increasing the concentration of the FMA beyond 6.52% and FMB beyond 0.62 % of the total working volume had a detrimental effect on the cell growth and protein production.

Along with the above mentioned tests, the amino acid consumption across different scales of bioreactor was also studied. The amino acids were divided into two groups: amino acid required for cell growth (glutamine, tyrosine, phenylalanine and isoleucine) and protein production (the remaining essential and non-essential amino acids). This provided an insight into the function of amino acids within the cells.

Key words: Chinese hamster ovary (CHO) cells, scalability, amino acid, monoclonal antibody

Kurzfassung

Der Markt für Biopharmazeutika hat sich von kleinmolekularen Arzneimitteln hin zu biotechnologisch hergestellten Therapeutika, wie rekombinanten Proteinen, kontinuierlich erweitert. Für die Herstellung dieser Proteine werden in großem Umfang genetisch modifizierte Säugetierzellen verwendet, vorwiegend die Eierstockzellen des Chinesischen Hamsters. Eine Herausforderung für die biopharmazeutische Industrie besteht darin, innerhalb eines begrenzten Kulturvolumens eine maximale Produktkonzentration zu erreichen. Dies wird durch die Intensivierung des Prozesses und das Verständnis der Grundlagen des Zellwachstums, des betreffenden Stammes, überwunden.

Das Hauptziel dieser Arbeit war die erfolgreiche Übertragung und Skalierung des Prozesses in verschiedenen Arten von Bioreaktoren mit einem Arbeitsvolumen von 15 mL bis hin zu 50 L. Die Prozessqualitätsattribute, wie die Konzentration an viablen Zellen und die Endproduktmenge, wurden zur Bewertung der Skalierbarkeit des Prozesses verwendet. Es zeigte sich, dass der Prozess in den verschiedenen Bioreaktortypen robust und skalierbar ist.

Der zweite Teil der Arbeit bestand in der Optimierung des Prozesses im Hinblick auf die Prozessparameter des gelösten Sauerstoffs und des pH-Werts. Durch Reduktion des Gelöstsauerstoffs auf 40 % und Beibehaltung eines pH-Werts von 7,1 konnte der Bedarf an reinem Sauerstoff in Bioreaktoren im Produktionsmaßstab verringert werden.

Für die weitere Prozessoptimierung wurden die Startkonzentrationen der Kohlenstoff- (Glukose) und Stickstoffquellen (Glutamin und Glutamat) zu Beginn der Kultivierung und deren Auswirkungen auf die Konzentration lebender Zellen und die Proteinproduktion untersucht. Die optimalen Anfangskonzentration von Glutamin und Glucose im Produktionsmedium liegen zwischen 0,6 bis 1,2 g/L bzw. 6 bis 12 g/L und damit etwas höher als bisher. Die Variation der Feedmedien ergab keine signifikante Verbesserungsmöglichkeit für den Prozess.

Bei der Untersuchung des Aminosäureverbrauchs in den verschiedenen Skalen des Bioreaktors wurde zwischen den Aminosäuren des Stoffwechsels und derProteinproduktion unterschieden. Einzelheiten dazu gaben einen Einblick in die Funktion der Aminosäuren innerhalb der Zellen.

Schlüsselbegriffe: Chinese hamsterovary (CHO) Zellen, Skalierbarkeit, Aminosäuren, Monoklonale Antikörper

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Abbreviation

°C	Degree Celsius
µg	Microgram
ccm	Cubic centimeter per minute
CHO	Chinese Hamster Ovary
ddH_2O	Double distilled water
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
FMA	Feed Medium A
FMB	Feed Medium B
g/L	gram per liter
HPLC	High performance liquid chromatography
IgG1	Immunoglobulin G
IVCC	Integral viable cell concentration
L	Liter
mAb	Monoclonal Antibody
MFCS	Multiple fermentor control system
mg	Milligram
mL	Milliliters
mM	Milli Molar
MTX	Methotrexate
N_p	Power Number
pCO_2	Partial carbon dioxide
pg/cell/day	Picogram per cell per day
PM	Production medium
Q_p	Cell specific productivity
Re	Reynolds number
RP HPLC	Reverse Phase-High performance liquid chromatography
rpm	Rotation per minute
TCA	Tricarboxylic acid

VCC	Viable cell concentration
vvm	Volume(gas) per Volume(liquid) per minute

1 Introduction and Goal

The global market of biopharmaceuticals in the year 2018 was greater than $ 250 billion and it has been continuously expanding. A shift from small molecular drugs to biotechnologically produced therapeutics such as recombinant proteins is observed in pharmaceutical companies. These alone are responsible for a revenue of more than $ 150 billion [1]. There has been a prominence of use of mammalian cells for the production of these pharmaceutical recombinant proteins. Amongst the 68 products produced by mammalian cells approved in the current period, 57 were produced by Chinese Hamster ovary cells (CHO) [2].

CHO cells are preferred due to their ability to grow in a suspension and thereby enable reproducible results in a serum free chemically defined medium [1, 2]. The other advantage of using CHO cells is the structural similarity of glycan from the product resembles that of the human monoclonal antibody (mAb). The end product of using CHO cells is higher recombinant protein yield and also high specific productivity due to gene amplification of the cells [3].

The CHO cells have proven to be advantageous in the biopharmaceutical industry, however, the limitations of the cell line must also be considered before the selection of the strain. One of the limitation is that the cells are susceptible to mechanical and shear stress due to absence of a defined cell wall. Therefore, during the cultivation, optimum process conditions such as, aeration rate and stirring speed, must be provided in the bioreactor to avoid any stress to the cells. This is achieved by optimization of the process parameters. The other method of optimizing the process is by further optimizing the medium in which the cells are cultivated and periodically providing the cells with depleted nutrients through feed medium.

During the course of this study the following key objectives were the prime focus of the project. These tests can be summarized as:

- Transfer and scalability of the process in different scale (15 mL to 50 L) and different types of bioreactors (single-use and multi-use Bioreactors)
- Optimization of process parameters in terms of dissolved oxygen concentration and pH variability.
- Alteration of the prime carbon and nitrogen source in the main production medium.

- Analysis of the consumed amino acid components during the entire course of the experiment.
- Altering the percentage of feed media added to the production bioreactor and testing the impact on cell growth and protein production.

2 Theoretical Background

CHO cell line (CHO K1) was first isolated by Dr. Theodore T. Puck in the year 1957 [4]. The isolated cell line was further modified in 1983, where *Urlaub et. al.* deleted the entire locus for the enzyme dihydrofolate reductase (DHFR) by mutagenesis, thus generating CHO-DG44 lineage [5]. In this cell line, the gene of interest (GOI) can be co-transfected with the functional copy of the DHFR gene. The selection of the cells that have the integrated DHFR and GOI can be further performed by growing the cells in a medium containing methotrexate (MTX) [6]. The compound, MTX, is a folate analog which is a direct inhibitor of dihydrofolate reductase and only the cells which have an increased copy number of DHFR are able to survive in presence of MTX.

Transfected cells in the presence of MTX exhibit one of the following conditions: decrease permeability to MTX, DHFR that is intrinsically less sensitive to MTX [7] or overproduction of DHFR activity. The last condition appears to be the most common and the overproduction is attributed to an increased synthesis of enzyme [6].

The DHFR enzyme is responsible for the formation of intracellular tetrahydrofolic acid, a cofactor that is essential for nucleotide biosynthesis and generation of glycine, purine and pyrimidine. The presence of MTX results in the growth of the cells that have been successfully transfected by the DHFR gene [6] and hence used for the preculture in the study.

In the current study, CHO DG44 is used to produce the protein of interest. In order to further optimize the process, the process parameters within the microenvironment of the cell along with the internal metabolic function of the cell must be studied.

2.1 Metabolism in CHO cells

Metabolism in CHO cells is characterized by a high rate of glycolysis and glutaminolysis [8, 9]. The main carbon and energy source for cell growth i.e., glucose and glutamine are directly correlated to the glycolysis and glutaminolysis respectively. It has been previously demonstrated by *Sanfeliu et. al.* that the cells cultured in media with high glucose and glutamine concentration exhibited higher uptake rate than those strictly controlled for their metabolism [10]. However, the by-products are to be considered, as large amounts of lactate and ammonium is accumulated during the cultivation from the conversion of glucose to lactate and decomposition and metabolism of glutamine to produce ammonium [11, 12].

It is essential to keep these by-products to a minimum level as they inhibit cell growth and protein production and can deteriorate the glycosylation quality of proteins [13, 14].

In some cell lines, lactate is produced during the initial growth phase. However, there is a shift in the metabolism as the cells move onto the next growth phase. The incomplete oxidation of glucose also results in accumulation of lactate even in presence of sufficient oxygen; this is a phenomenon known as "Warburg effect" or aerobic glycolysis [15, 16]. This accumulation of lactate is detrimental for cells and it can be lowered by simultaneously substituting the carbon source to both glucose and galactose [17]. A culture cultivated in presence of both glucose and glutamate undergoes a metabolic shift and is capable of metabolising lactate. However, use of galactose results in slower metabolism and in turn diminished specific growth rate [18].

Apart from glucose, glutamine also has an important role in modulating the initial lactate production in different cell lines [19]. It is one of the key sources of energy used during the tricarboxylic acid (TCA) cycle in fast growing cells. However, this might lead to a shorter cycle with oxidation of glutamine to the intermediate malic acid, which is then turned into pyruvate, and finally into lactate, in the cytosol [6]. This alternative pathway is known as glutaminolysis.

During the cell proliferation, the growth phase is characterized by high specific rates of glycolysis and glutaminolysis and, as a result, a high lactate production rate. The metabolism shifts towards an effective substrate consumption during stationary phase, represented by higher flux through TCA cycle, lower metabolic flux through glycolysis and often lactate consumption [20].

The other by-product to be kept in lower concentration is ammonium. Metabolism of glutamine is the main source of ammonia production. Glutamine in the medium is hydrolyzed to glutamate and ammonia. Glutamate is further hydrolyzed via glutamate dehydrogenase to α-ketoglutarate and another molecule of ammonia. Thus, the accumulation of the ammonia can be reduced by substitution of glutamine by glutamate.

Over the last few decades, apart from studying the role of glucose and glutamine in metabolism, a lot of research has been done to study the metabolic flux analysis within CHO cells. *Sen Gupta et. al.* [21] and *Templeton et. al.* [22] used isotopic labelling in combination with metabolic flux analysis. Both studies reported higher metabolic flux in stationary phase through the pentose phosphate pathway (PPP) and TCA cycle. Furthermore, *Templeton et.*

al. showed a direct correlation between high PPP and TCA cycle activities and monoclonal antibody (mAb) production. Furthermore, *Wahrheit et. al.* suggested that the glycolytic regulation controls the metabolic shift in different CHO cultivations. These studies indicate the increase of production of protein during the stationary phase and this is studied in this research to understand production of IgG1 in CHO DG44 cells.

2.1.1 Amino acid

Along with carbon and nitrogen sources, cells also require vitamin, amino acid and other nutrients in the medium. Depletion of any component will negatively influence the cell growth and protein production. Amongst these vital components, amino acids play an important role in the cell growth and the titre production [23, 24]. They are an important source of nitrogen and foundational building blocks of both native and recombinant proteins. They are also involved as intermediates in several metabolic pathways.

Apart from having an influence on the different metabolic functions in mammalian cells, amino acids also have the potential to act as buffers for intracellular pH as they exist as zwitterions at physiological pH [25].

These have been extensively studied to further improve the overall performance of the cells, in terms of both increased cell growth and titre production; such as, addition of threonine, proline, and glycine improved CHO cell growth by influencing metabolic parameters such as glucose and glutamine consumption along with reduction of lactate and ammonium production [13]. Additionally, cell growth was enhanced when media was supplemented with proline, asparagine and serine [18]. It has also been demonstrated that glycine and asparagine protect the CHO cells at elevated pCO_2 conditions.

According to *González-Leal et. al.*, leucine and arginine had the highest positive and negative impact on the cell viability, respectively. The growth rate was negatively affected by leucine and threonine and the mAb concentration was positively influenced by the presence of valine and Arginine [26]. These are a few of the influences of amino acid in the medium for cell growth.

Amino acids also help in regulation of the ammonia concentration in the media. As mentioned earlier glutamine is the main source of ammonia production and this can be reduced by substituting glutamine with glutamate. Glutamine can also be hydrolyzed to glutamate and ammonia. Alternatively, glutamate and pyruvate can react to form alanine

and α-ketoglutarate which in turn can reduce the ammonia toxicity by redirecting the amine group from glutamate to alanine.

The suitable concentration of amino acid in medium varies depending on the cell line, and desired protein. A comprehensive study of the amino acid consumed during the course of the cultivation could open up the possibility to optimize the medium used and improve the feeding strategy.

2.1.2 Factors influencing the metabolism of the cells

Influence of pH: Process parameters such as dissolved oxygen [DO] concentration, pH, temperature, agitation and aeration have influenced cellular metabolism.

Lactate production is the by-product of one of the metabolic pathways and a linear correlation of specific lactate production rate and set point of pH and temperature has been observed in batch cultivation [27, 28]. On studying this further, a metabolic switch to lactate uptake has also been shown to be dependent on pH set point [29, 30].

Depending on cell type and clone, the lower critical pH limit in mammalian cell was measured to be between 6.6 to 6.8 [31]. pH has also been associated with interfering with product quality [32] and impair cell proliferation [31]. In industrial process, it is important to ensure production of lactic acid is controlled to a minimum concentration, this accumulation of lactic acid can be restricted by running the cultivation at low pH at the risk of inhibiting cellular proliferation while an alkaline pH has an opposite effect on the cultivation [33–35]. It was also hypothesized by *Konakvsky et. al.* [33] that higher pH results in higher carbon flux and in turn lead to more cells which produce more product.

Both alkaline and acidic parameters not only influence the overall metabolism but also cell growth and productivity [30, 32, 36].

The pH in this cultivation was maintained by aerating the bioreactors with CO_2 gas (pH also increased after addition of FMB but was controlled using CO_2 gassing). Higher levels of pCO_2 (>105 mmHg) have shown inhibition in growth of CHO cells and have drastically reduced the productivity of the cells [37]. *Gray et. al.* [37] found the optimum level to maintain CO_2 in CHO cell cultivation to range between 30 to 76 mmHg.

Influence of oxygen: Oxygen plays a major role in cell survival and has a dual function in the culture medium. It serves as the terminal electron receptor in oxidative phosphorylation and

it also forms toxic free radicals that can damage or destroy critical cellular components such as DNA, protein and lipid [38]. This leads to an overall cell damage and poor growth of mammalian cells.

The cells may adapt to hypoxic condition where cells are able to grow despite low oxygen concentration by induction of specific antioxidant enzymes [39, 40] While designing a CHO cell cultivation process, this dual nature of oxygen can be a critical issue [41].

The problem of oxygen delivery is of a major concern during scaling up of the process. This can be overcome by reducing the cell demand for oxygen. In this case, the oxygen consumption must be decreased below the critical oxygen tension. Operating it at lower oxygen concentration is also advantageous with respect to limiting the level of oxygen driven free radicals, thereby protecting the cellular components [41].

The impact of dissolved oxygen [DO] and pH on final product was examined in this study.

2.2 Growth characteristics in the cell

During the cultivation, the cells undergo different phases. These have been depicted in Figure 1**.**

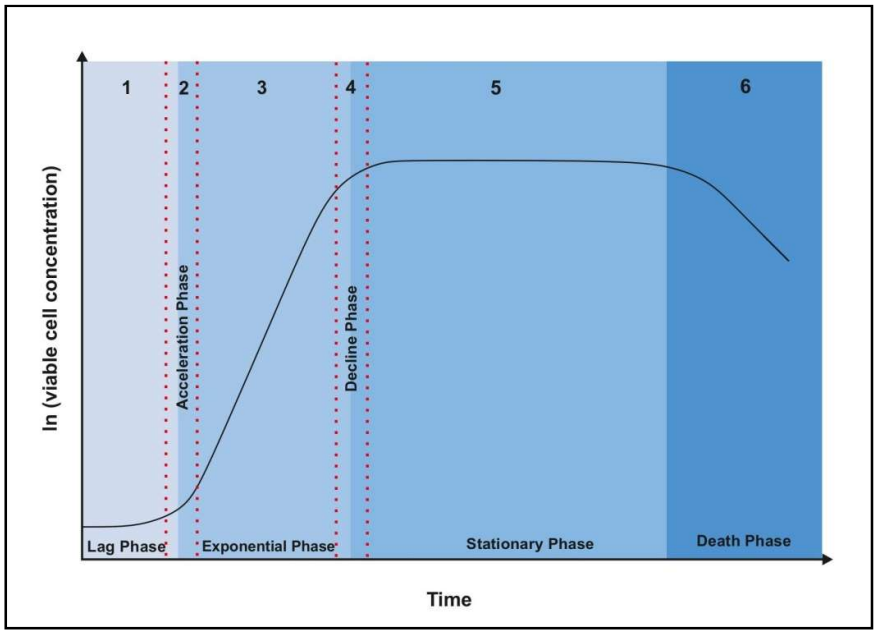


Figure 1: Growth curve of microorganism

Phase 1 indicates the lag phase. When the cells are initially inoculated in the bioreactor, they require time to adapt to the new environment, resulting in slow growth rate. In phase 3, the cells adjust to the new conditions and growth rate increases. This is known as the

exponential phase. This phase is characterized by high glycolysis and glutaminolysis and in some cases results in higher lactate production rate. The growth rate can be measured as the change in cell concentration per unit time.

$$\mu = \frac{1}{x} \cdot \frac{dx}{dt} \quad (1)$$

Where

x : Cell concentration [cells/mL]

μ : Specific growth rate [h^{-1}]

t : Time [h]

Phase 5 represents the stationary phase. During this phase, the replicating cells are in equilibrium with dying cells. Also, the metabolism shifts towards a more effective use of substrate and is represented by a lower metabolic flux through glycolysis, a higher flux through TCA cycle and often consumption of lactate.

The final stage represented in the figure is the death phase (phase 6), during which, the cells lose viability or are destroyed by lysis.

The specific growth rate in the transitional phases; namely, acceleration (phase 2) and decline phases (phase 4) are dependent on the concentration of the nutrients in the medium. The concentration of a single substrate can influence the growth rate; this component is known as the growth limiting substrate.

During a balanced growth, the growth rate is correlated to the concentration of growth limiting substrate and can be represented by the Monod equation,

$$\mu = \frac{\mu_{max} \cdot S}{K_S + S} \quad (2)$$

Where

S : Growth limiting substrate concentration [g/L]

K_s : Substrate constant / Monod constant [g/L]

μ_{max} : Maximum specific growth rate [h^{-1}]

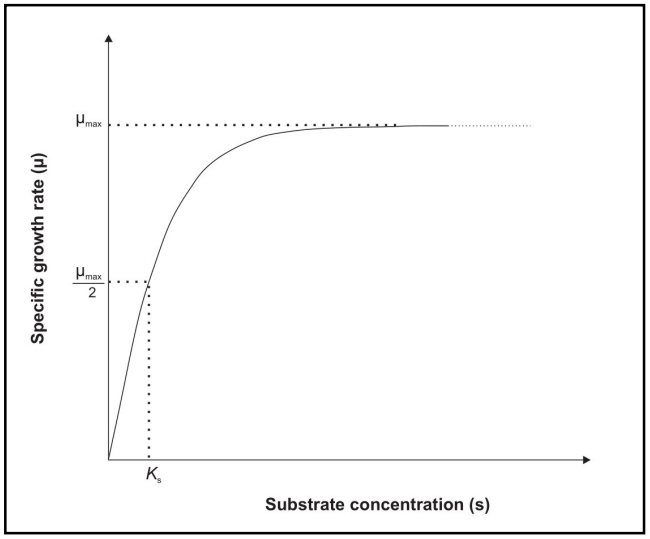


Figure 2: Monod equation curve: Relationship between the specific growth rate and the concentration of growth limiting substrate in cell culture

Table 1: Cell growth phases (modified [47])

Phase	Description	Specific growth rate
Lag	The cells adapt to new environment	$\mu \approx 0$
Acceleration	Growth of the cell begins	$\mu < \mu max$
Exponential	Maximum growth rate attained	$\mu \approx \mu max$
Decline	Slow growth rate due to exhaustion of nutrients and accumulation of inhibitory by-products	$\mu < \mu max$
Stationary	No significant change in growth rate	$\mu = 0$
Death	The viability of the cell decreases	$\mu < 0$

The transitions between these phases are a result of change of the culture condition such as nutrient depletion and secondary metabolite accumulation. Phase 4 (Figure 1) determines the maximum viable cell concentration (VCC) as this indicates the shift from exponential phase to the stationary phase; whereas the culture longevity is determined by the transition between the stationary phase and the death phase. These collectively determine the integral of viable cell concentration (IVCC). Protein production by in-vitro mammalian cell cultivation is also directly correlated with culture longevity and IVCC [23, 42].

IVCC is used for comparison of the different process conditions and allows for different scales of bioreactor to have a common factor of comparison.

2.3 Kinetics of cell growth and product formation

Cell culture is characterized by cell growth, nutrient consumption and desired protein production. Cell culture kinetics can be quantitatively described based on the concentration of components, activity parameters and stoichiometric ratios.

Under different metabolic conditions, cells utilize different nutrients and produce different concentrations of by-products. Any alteration in metabolism can be elucidated by modifying stoichiometric ratios of media components.

An accurate estimation of the specific rates is vital for bioprocess optimization. A comparison of the cell line and cultivation conditions can be quantified using cell specific rates [43].

In 1975, S. J. Pirt [44] discussed the kinetics of growth linked and non-growth linked product formation in microbial cultures. Growing cells produce primary metabolites which are classified as growth linked products, while non growth linked products are considered equivalent to secondary metabolites.

The growth linked products are described by the equation

$$\frac{dp}{dt} = q_p \cdot x \qquad (3)$$

Where;

p	: Product concentration	[g·L^{-1}]
q_p	: Specific rate of product formation	[g·cells^{-1}·mL^{-1} ·h^{-1}]
t	: Time	[h]

The product formation is related to the biomass produced.

$$\frac{dp}{dx} = Y_{p/x} \qquad (4)$$

Where $Y_{p/x}$ is the yield of product in terms of biomass

The specific rate of formation of a product can be correlated to the growth rate and the yield of the product. In order to do so the equation 4 is multiplied with dx/dt.

$$\frac{dx}{dt} \cdot \frac{dp}{dx} = Y_{p/x} \cdot \frac{dx}{dt} \quad (5)$$

$$\frac{dp}{dt} = Y_{p/x} \cdot \frac{dx}{dt} \quad (6)$$

Substituting dx/dt by μ·x from equation 1 in the above equation 6, we get

$$\frac{dp}{dt} = Y_{p/x} \cdot \mu x \quad (7)$$

Substituting equation 3 in equation 7, we get a correlation between the specific rate of production and yield of product in terms of growth rate

$$q_p = Y_{p/x} \cdot \mu \quad (8)$$

A modification of equation 3 was used to calculate the cell specific substrate consumption and by-product production during the course of cultivation. For example:

Consumption of glucose was measured as:

$$d_{glu} = \left(\frac{Glu_2 - Glu_1}{t2 - t1}\right) \cdot \left(1 / \frac{VCC_2 + VCC_1}{2}\right) \quad (9)$$

Where

d_{glu} : Glucose consumption [g·day^{-1}·cell^{-1}]

Glu_1 : Glucose value at time 1 [g·L^{-1}]

Glu_2 : Glucose value at time 2 [g·L^{-1}]

VCC_1 : Viable cell concentration at time1 [10^6 cells·mL^{-1}]

VCC_2 : Viable cell concentration at time2 [10^6 cells·mL^{-1}]

Equation 9 is applicable for estimation of values for protein and lactate production, glutamine and glutamate consumption. This is used to compare the results in Section 3.

2.4 General aspects of a bioreactor

High value biopharmaceuticals such as monoclonal antibodies or insulin are produced using mammalian or microbial expression systems [45]. This is achieved by cultivating the transfected cells in an optimum environmental condition provided by a suitable bioreactor.

A bioreactor is a defined space which provides the possibility for a biochemical reaction to

take place aided by a biocatalyst (e.g. cells). Desired products such as biomass and expressed proteins are converted using these biocatalysts.

The main function of bioreactor is to provide cells with suitable conditions for growth and production of the desired protein. When designing a bioreactor, it is important to ensure that the vessel is capable of being operated aseptically for the complete duration of cell growth. It is also vital to thoroughly mix the nutrients and gases within the culture medium. Thus, adequate aeration and agitation should be provided to meet the metabolic requirements. Along with aeration and agitation, maintaining optimum pH and temperature are requisites for efficient growth of the microorganism. The designed reactor should be capable of providing precise control of these parameters.

Once the basic design of a bioreactor is finalized, a scaled-up and scaled-down version of bioreactor needs to be built. This ensures that cultivation can be carried out across different scales and reproducibility of results can be tested. One of the criteria to facilitate successful scale-up and scale-down is the geometric similarity between these bioreactors [46]. Certain criteria are kept constant while scaling up and scaling down the bioreactor dimensions. These criteria include bioreactor geometry, volumetric mass transfer coefficient, volumetric power input, volumetric gas flow rate and mixing time [47].

Traditionally small bioreactors such as shake flasks, T-flasks, spinner and roller bottles have low level of instrumentation and control. In order to ensure cell growth in these reactors, they are placed on external equipment such as shakers and incubators. In contrast to these, stirred tank reactors are equipped to provide large volumes of mass and heat transfer rates and excellent mixing facilities.

Several bioreactors have been developed over the last six decades predominantly; stirred tank, airlift and bubble column bioreactors [48]. A minimum of 70% of processes in the biopharmaceutical industry use stirred bioreactors with a maximum working volume of 70 m^3 for microbial cells and 25 m^3 for mammalian cells. These include both multi-use and single us bioreactors [49].

In the last decade, there is an increased interest in the field of disposable single-use systems. One of the major advantage of using the single-use stirred bioreactor is that the pre-sterilized single-use bioreactors help in reducing the costs associated with sterilization, cleaning and maintenance of the bioreactors [50]. The monetary efficiency of processes utilizing single-use bioreactors has increased due to shorter change over times and can lead

to 54% lower capital cost [51].

A major problem in the biopharmaceutical industry is the product cross contamination. Production yield can be reduced by unwanted contamination and would require additional purification steps. Since the bioreactor is replaced after each batch, the risk of contamination between the different batches is eliminated using single-use systems.

Of the traditional small scale bioreactor, disposable culture flasks have been used for several years in the industry. These are, however, confined to early inoculum stages in the cell expansion. The “wave” reactor launched in 1996, was the first commercially available single-use bioreactor and was used to culture animal cells [52]. Since then the single-use technologies have been increasingly accepted and adapted by the biopharmaceutical industry. The first single-use stirred tank bioreactor had a working volume of 250 L and was launched by Thermo Fisher Hyclone in the year 2004.

Single-use bioreactors have come a long way since their introduction nearly six decades ago. Contemporary bioreactors allow users to select between a wide range of different impeller types and aeration devices. Users also have access to relevant engineering data such as mixing time, volumetric mass transfer rate and residence time distribution [49]. This allows for identification of potential limitations of certain bioreactor design, enabling the improvement of the process efficiency, facilitating comparison between other bioreactor types and allowing rapid scaling up and scaling down. Initially used to reduce the development time when establishing a cell line, medium and process conditions, single-use bioreactors are now regarded as well established in preclinical as well as clinical production of the desired products. Products such as therapeutic monoclonal antibodies and vaccines are typically produced in single-use bioreactors [53].

However, single-use technology has several inherent limitations. One such drawback is the disposal of the bioreactor bag. Biopharmaceutical companies are confronted with the arduous task of disposal of large volumes of solid waste and this further increases the running cost [50, 50, 51]. Another important aspect to be considered is the quality of the material used. A suitable material must be free of the substances that can potentially migrate into the culture medium, hindering the cell growth and viability or product production [50]. These chemicals are often referred to as extractable and leachable. Intensive research is being carried out in this field to overcome the limitation of chemical composition of single-use bioreactors [50, 51].

The single-use bioreactors represent a comparatively younger technology compared to the stainless steels cultivation system. Due to the increase in demand of the single use technology for the cultivation of seed and main culture, many different disposable bioreactor concepts have been developed. The commercially available single-use bioreactors range from a working volume of 10 mL up to 4000 L [53]. These reactors differ in geometric shape, mixing technology and material. The bags either consist of a rigid container or a flexible bag usually made up of polyethylene, polystyrene or polypropylene [53].

On the other hand, rigid systems are made up of polycarbonates and do not require a support container. Due to the rigid properties, they are not capable of folding which in turn could lead to layer breakup followed by leakage in the bioreactor [53].

In case of flexible bags, it is important they are installed correctly to achieve optimum performance in terms of heat transfer. The largest available capacity of the single-use bioreactor is limited to 4000 L. In order to increase the production of the final product; the focus has shifted to producing high titre of the desired protein within a limited volume of culture [50, 53].

The control of aeration and mixing in these reactors also varies and is either by wave-mixing, orbital shaking or stirring. While the wave mixed bioreactors predominate the production of seed culture, stirred tank reactors are used for production scale [45].

Although efforts have been made to characterize the engineering parameters in the different types of single-use bioreactors, scale up from small bioreactors to production scale bioreactors is still challenging [54, 55].

Before studying the scale up and scale down parameters, it is important to understand the different mode of operations in the bioreactors.

2.5 Operating modes of bioreactor

In the design of the biotechnological production process, selection of the operation mode is just as important as the selection of the type of bioreactor. The operation of a bioreactor is classified as discontinuous (batch or fed batch) and continuous mode (perfusion or chemostat). The mode is selected based on the type of cell line used, rate of substrate consumption, secondary metabolite production and the desired product yield.

2.5.1 Batch process

A batch process is a closed system with constant volume maintained throughout the process. All the nutrients required for the cell growth are added in the beginning of the process and no addition occurs after the bioreactor has been inoculated with the exemption of air or oxygen for aerobic cultivations, acid or base for pH control and antifoam solution to reduce the foam produced.Figure 3is a schematic diagram of batch phase where A is the component added to the reactor system aseptically. These include medium, inoculums, acid, base and antifoam.

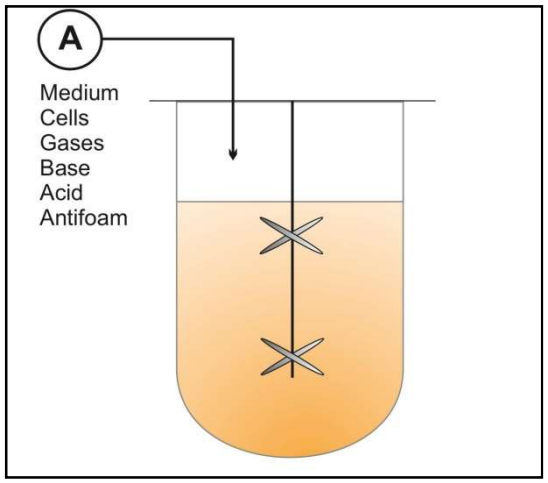


Figure 3: Schematic representation of batch process

Batch processes are widely used for its simplicity. Before reaching production scale, cell expansion is carried out as batch cultivation. The series of batch cultivation until the start of production scale is called seed train. During expansion, the cell culture is transferred to a fresh medium while the cells are rapidly growing. If the cell culture is not transferred to a new medium periodically, the growth would be inhibited by accumulation of metabolites and the limitation of substrates.

One of the drawbacks of batch cultivation is the inability to produce high concentration of desired product due to lower integral viable cell concentration. Another drawback is nutrient depletion and accumulation of secondary by-products limiting the longevity of the cell growth in batch process. This can be overcome by addition of substrate or product precursor to reach higher cell and product concentration. This paves the way for continuous and fed-batch cultivation.

2.5.2 Continuous Process

In a continuous process, the feed is continuously supplied to the system and the product is streamed out of the bioreactor. The process is subdivided into two categories, namely, one where the cells are retained and the supernatant is removed (e.g.: perfusion) and the other where a certain concentration of the cell along with the supernatant is also removed (e.g.: chemostat or turbidostat).

In these cases the growth rate can be maintained at a constant concentration by regulating the flow rate of A and B (Figure 4). The cells can be sustained in exponential phase to attain maximum biomass yield and protein production.

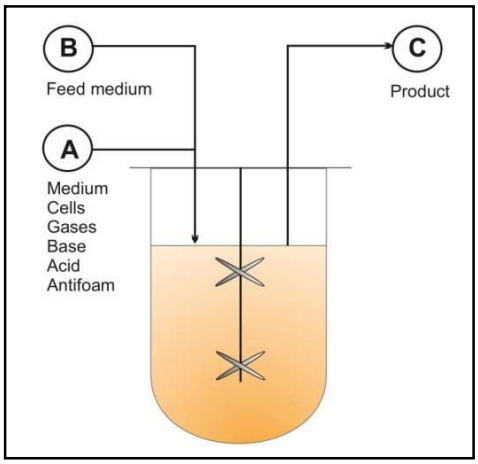


Figure 4: Schematic representation of continuous process

This mode is optimum for the production of higher concentration of desired product and the reactor can be functional for a long period of time without being shut down. Also, the amount of product extracted from the process can be increased by varying the duration of the cultivation. However, it is not commonly practiced in mammalian cells unless in conjugation with cell recycle.

2.5.3 Fed batch process

To overcome the shortcomings of batch and continuous process, fed batch process is used where the cell growth and protein production increases through periodic addition of required nutrients. Currently, fed-batch mode is the most frequently used process strategy in an industry as it combines the operational safety of a simple batch process with high productivity of continuous fermentation [56].

In fed-batch process, the culture system is initially less than the total working volume and

the process is run in batch phase. The feeding starts before the substrate is completely consumed or has reached the growth limiting values. The main advantage of this process over the batch process is the significant improvement in the growth phase along with higher cell concentrations and product yield.

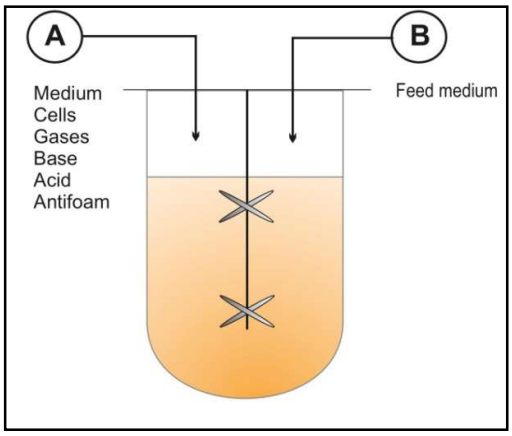


Figure 5: Schematic representation of fed-batch process

This operational mode is extensively used in the pharmaceutical industry due to high IVCC and increased protein concentration.

In the current work, the cultivations run in a fed batch process with the bioreactors being filled with 66% of the total working volume. The supplement of feed starts day 3 onwards and is fed every day until the end of the cultivation.

2.6 Process development and screening systems

After understanding the cell metabolism, growth kinetics and operation mode of bioreactors, the next step is to understand the process development and screening of the system.

The process development and optimization can be divided into four phases: (1) strain construction and optimization, (2) screening of cultivation conditions such as temperature, pH, media, etc., (3) process development of technical scale such as gassing, agitation, feeding strategy, and (4) scale up to production bioreactors.

Process development does not solely consist of optimizing process parameters but also includes gaining a comprehensive understanding of the production process. This involves identifying the parameter boundaries within which the process and product qualities are optimal [57]. Figure 6 represents the different optimization area and lists the important parameters schematically [58].

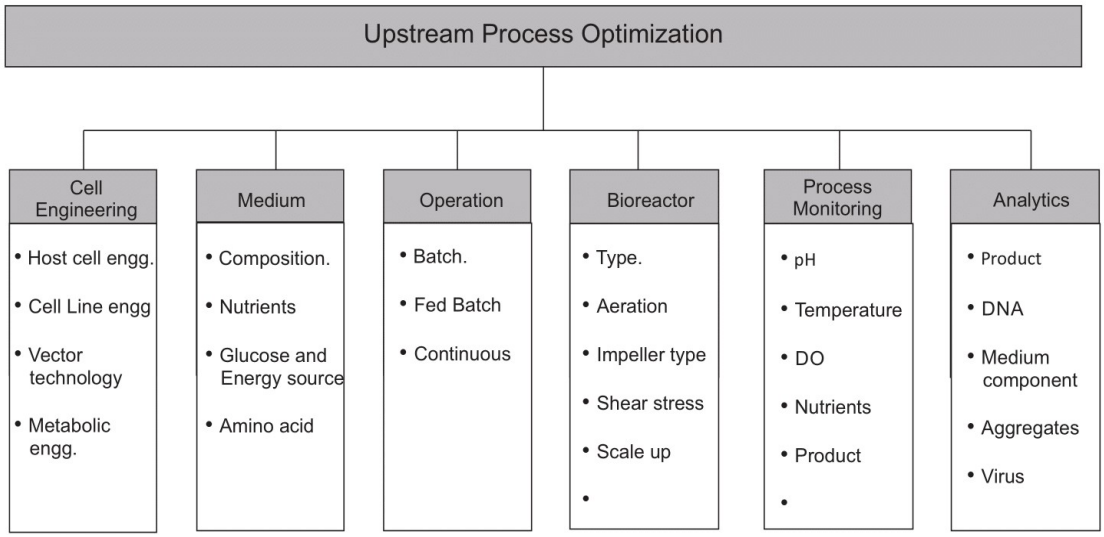


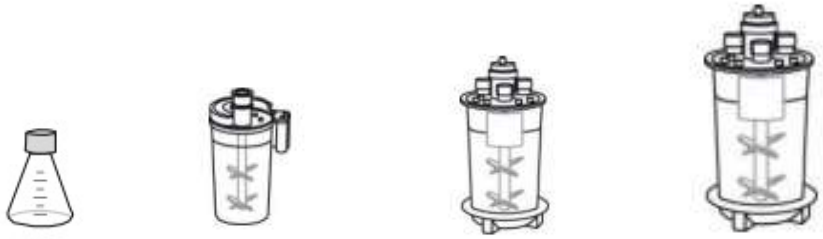
Figure 6: Important parameters for upstream process optimization

As per Figure 6, optimization of upstream processes can be divided based on various parameters. The optimization is initiated at cell line development where the genetic structure of the organism can be altered to produce the desired product. Next the medium used for the culture can be studied, in order to provide the cells with sufficient nutrients for sustenance and proliferation. Also the system in which cells grow should provide sufficient oxygen transfer, heat dissipation power and acceptable mixing characteristics. Additionally, the system should provide a possibility to monitor and control important parameters such as temperature, DO and pH online. The influence of all these changes can be validated by the analytics mainly the protein production.

Along with the above mentioned parameters, scalability and reproducibility of the process is also important for the production of desired protein. Therefore, a high degree of parallelization along with process transfer to larger scale for production are beneficial for the process development system. In the last decade, new high throughput methods have accelerated the strain development [59] and this has paved the way to development of new technologies.

In the following table, the summary of the technologies used currently for the research are enlisted.

Table 2: Summary of technology used for this research



Scale	Shake Flask	Small-scale bioreactor	Laboratory scale bioreactors	Production scale bioreactor
Working volume	5 mL – 3 L	10 mL – 500 mL	1 L – 5 L	10 L – 4000 L
Mixing technology	Shaken	Stirred	Stirred	Stirred
Process parameter monitoring and control	Partially monitored	Yes	Yes	Yes
Automation	No	Yes	Yes	Yes
High throughput	Yes	Yes	No	No
Material	Multi- or single-use	Multi- or single-use	Multi- or single-use	Multi- or single-use
Main application	Screening	Screening and process development	Process development	Production of desired product

2.6.1 Shake flask

Shake flasks have been traditionally used for multi-parallel cultivations of both microbial and mammalian cell cultivation [60]. The capacity of shake flasks range from 25mL to 5 litres and are used for a wide array of purposes and can be modified with or without baffles and can be multi or single-use [61]. Currently, they are used for biomass generation and seed cultures [59].

One of the drawbacks of the shaker flask is that they must always be used with external systems to provide the culture broth with desired temperature and agitation. These flasks are placed in incubator shakers and the shaking movement is responsible for mass transfer

and mixing in the flasks. This shaking can be performed using either orbital motion or linear reciprocating motion [61]. The most commonly used shakers are the orbital shakers.

The use of shake flasks for process development is limited due to the constraints with respect to the oxygen transfer, automation and on-line process monitor and control of critical variables. Additionally, the maximum working volume is only 10-20% the nominal volume resulting in limitation of the scale-up in these flasks [62].

Several efforts have been made to increase the process monitoring and control in shake flasks. They, however, remain inferior to the stirred tank reactor [63]. To overcome the disadvantages of shake flask, automated micro bioreactors have been developed which can be used for both screening and process development.

2.6.2 Stirred tank bioreactor

Demands for decreasing the timeframe for cell culture process development has been an important objective driving the development in biopharmaceutical industry. In order to speed up the development, disposable automated micro-scale bioreactors are gaining an increased interest due to its advantages including high automated control, high throughput capacity and short turnaround time [64].

Small scale bioreactors are available in volumes ranging from 10 to 500 mL [65]. These small scale bioreactors are systems that replicate the functionality of laboratory scale bioreactors. The process parameters are monitored and controlled using the latest technology in the market. These are extensively used for Design of experiments (DoE), process variation, process optimization and development.

These systems are advantageous in comparison to micro-titre plates due to presence of larger volume which can be further used for comprehensive measurement of off-line analytics. Another advantage is the extensive on-line monitoring for every individual micro-bioreactor in terms of testing different parameters within a given time frame.

Addition of liquids by pumping or liquid handler is another benefit of the small scale bioreactors. Different feed profiles, antifoam, chemical for pH correction or inducer substances can be added to the bioreactor. However, the complexity of the system increases with every additional liquid that has to be pumped in since they have to be set up in a manifold for each of the parallel bioreactors.

The miniature stirred tank bioreactors were designed to pose as scale-down models of

production scale cultivation systems and comparison of the process is conducted to test the reproducibility of the same across the different scales.

Laboratory scale bioreactors and production scale bioreactors have similar advantages to that of the small scale bioreactor in terms of mixing technology, automation, process parameter monitoring and control.

Laboratory scale bioreactor ranges from 1 L to 5 L and can be single-use or multiuse. These are mainly used for process development as the volume for production is attainable in small scale laboratories.

Production scale bioreactors on the other hand are mainly used in pharmaceutical companies. The volume ranges from 10 L to 4000 L and is mainly used for the production of the desired product.

The presence of extra ports in the laboratory scale and production scale bioreactors is advantageous for users who want to employ spectrometers to analyse the changes that take place during the cultivation in the bioreactor. UV-Vis, NIR, MIR, Raman, Viamass sensor, Trace sensors for online glucose and lactate measurement can be inserted into the bioreactor and the data can be collected using corresponding software. However, they both are unable to be used for high throughput cultivation.

2.7 Scale-up and scale-down of cell culture process

Scale-up and scale-down model development relies on engineering principles where the parameters can be subdivided into two categories: scale-dependent and scale independent [66]. The scale independent variables remain constant across different scales of the bioreactor. These include pH setting, temperature, dissolved oxygen setting, inoculation cell density and cell age. The scale-up/down of the process is based on scale-dependent parameters such as volumetric mass transfer coefficient (k_La), tip speed, power per unit volume (P/V), and volumetric flow rate (vvm) [67, 67–69].

Table 3: Parameters to be considered for scale up and scale down of a process

Parameter	Importance of the parameter
Oxygen transfer rate (OTR)	Higher oxygen transfer rate results in an improvement in the process if the OTR from gas to liquid is rate limiting for a specific process [70, 71]
Volumetric mass transfer coefficient (k_La)	The oxygen transfer rate within the reactor improves with the increase in the k_La value[64, 70–72]
Dissolved oxygen (DO)	The driving force for oxygen transfer increases with high DO values[73]
Volumetric flow rate (vvm)	A higher airflow rate results in an increase in the k_La and oxygen transfer rate [64, 74]
Power per unit volume (P/V)	Higher P/V ratio helps to improve the k_La and mixing in the reactor but it may also damage the cell morphology [64, 74, 75]
Tip Speed	The morphology of the cell must be considered while scaling up in terms of tip speed. High impeller tip speed may result in increased shear stress leading to cell damage and decrease in viability [76, 77]

Volumetric mass transfer coefficient (k_La) is influenced by changing the vessel geometry, stirrer type, gas bubble residue time and baffles. The coefficient is derived from surface area of the gaseous phase and is directly correlated to the oxygen transfer rate. The larger the k_La, the higher the aeration capacity of the bioreactor [78]. The correlation of the volumetric mass transfer coefficient can be described by equation 10.

$$OTR = k_L a \cdot (c^*_{O_2} - c_{O_2}) \quad (10)$$

Where

OTR	:	Oxygen transfer rate	[mmol·L^{-1}·h^{-1}]
k_La	:	Volumetric mass transfer coefficient	[h^{-1}]
$c^*_{O_2}$	:	Oxygen saturation concentration	[mmol·L^{-1}]
c_{O_2}	:	Oxygen concentration (Liquid phase)	[mmol·L^{-1}]

The most common method of scaling of the process is by maintaining the geometric similarity of the bioreactors [79]. A simple strategy to scale up or scale down is to maintain the height to diameter (H/D) ratio. Typical bioreactors have a H/D ratio between 2/1 and 3/1. Other properties to be considered while scaling of the process are the ones that are affected by the mass and heat transfer along with flow behaviour or mixing conditions.

In order to describe the flow behaviour in a stirred bioreactor, two dimensionless quantities can be used, namely, Newton number and Reynolds number.

The Newton number is dependent on power input and can be described by the equation 11. It is the ratio of external force exerted (P) to the inertial force imparted to the medium.

$$N_p = \frac{P}{\rho \cdot n^3 \cdot {d_r}^5} \tag{11}$$

Where

N_p:	Power number	[-]
P:	Power	[W]
n:	Agitation rate	[s^{-1}]
d_r:	Impeller diameter	[m]
ρ:	Medium density	[$Kg \cdot m^{-3}$]

The Reynolds number for a stirred tank reactor is as described in equation 12. It is only dependent upon the agitation rate, the impeller diameter and kinematic viscosity.

$$Re = \frac{n \cdot {d_r}^2}{v} \tag{12}$$

Where

Re:	Reynolds number	[-]
v:	Kinematic viscosity	[m^2/s]

Rushton et. al. [80] demonstrated that power number was related to the Reynolds number by equation:

$$N_p = c(Re)^x \tag{13}$$

Where

c: Constant dependent on vessel geometry but independent of vessel size.

x: exponent based on type of flow

By subsisting equation 13 with Reynolds number and power number:

$$\frac{P}{\rho \cdot n^3 \cdot {d_r}^5} = c\left(\frac{n \cdot {d_r}^2}{v}\right)^x \quad (14)$$

The three different flow regimes can be studied using the two dimensionless variables and are demonstrated in Figure7 [81].

1. For Re<20, a laminar flow prevails in the stirred liquid. In this regime N_p is inversely proportional to Re and the power absorbed in this region is a function of viscosity of the liquid. The x value is equal to the slope of the graph and is estimated to be -1.
2. For 20 < Re < 5 x 10^4, a transitional zone exists where there is no consistent relation between the Reynolds number and power number. The value of x is a variable depending upon the slope of the curve in the transient / transition zone.
3. For Re > 50 in baffled and Re > 5 x 10^4 in unbaffled vessels, the flow is turbulent and the N_p value remains constant; independent of the Reynolds number. The value of x in equation 13 is zero for this zone.

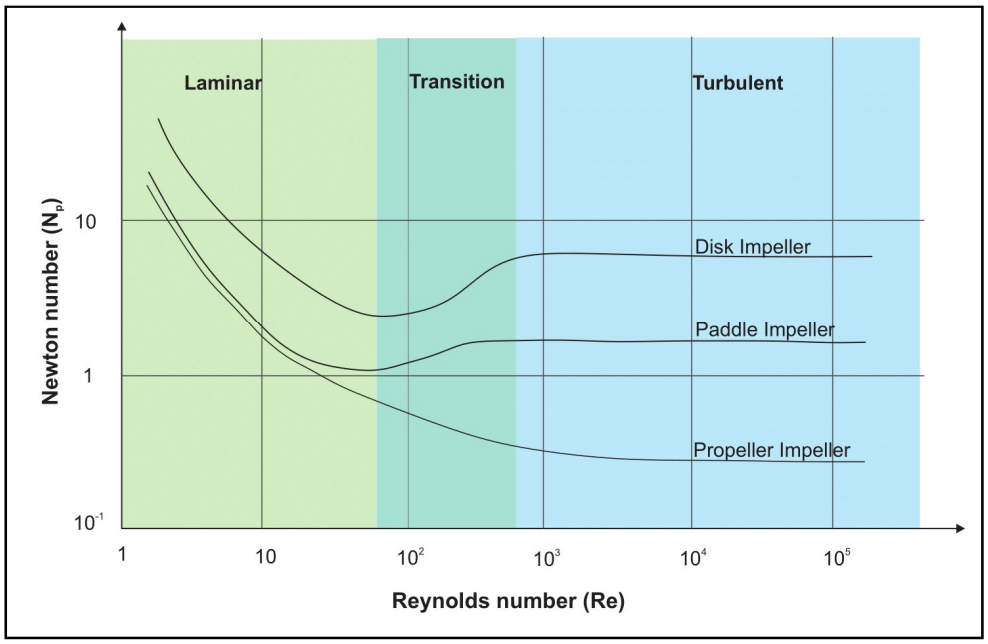


Figure7:Characteristics of different impeller in laminar and turbulent flow regime.

(As illustrated by Chmiel [82])

In order to obtain an optimal mixing performance, the bioreactors are operated in the turbulent flow regime [83]. The Newton number within the turbulent condition is dependent on the type, number and distance of impellers as well as the configuration of the vessel. Therefore, it is easily producible across different scales of bioreactors.

3 Results and Discussion

To evaluate the scalability of the process in different volumes, CHO DG44 cell line was used. The cryopreserved vials of the cell line was thawed to room temperature and re-suspended in warm seed culture medium. The cells were passaged after 3 days, maintaining an initial viable cell concentration of 0.2 x 10^6 cells/mL. The preculture step was carried out in 0.5 L non baffled shake flask and was incubated in an incubation shaker at 36.8°C, 7.5% CO_2, 85 % humidity and a shaking rate of 120 rpm (Shaker diameter: 19 mm). The culture was passaged five times before being transferred into the bioreactors. The detail of the preculture is described in the appendix 5.1.

The cultivation was carried out in multi-use bioreactor, namely, 2L glass bioreactor and 10 L stainless-steel bioreactor and single-use bioreactors, viz., 500 mL shake flask, 15 mL automated micro bioreactors (AMBR15), 250 mL automated micro bioreactors (AMBR250) and 50 L single-use bioreactor (SUB).

The main cultivation was inoculated with an initial viable cell concentration of 0.3 x 10^6 cells/mL and the total duration of the fed batch cultivation was 12 days. The two type of feed, Feed medium A (FMA) for macronutrients and Feed medium B (FMB) for micronutrients were fed every 24 hours after a batch phase of 72 hours. The harvest was collected on Day 12 and processed further for downstream processing.

The process parameters were maintained at a constant range for the different scale. The temperature was controlled at 36.8°C. The DO was maintained at 60 % using the gas mix of air, nitrogen and oxygen with a constant gassing rate of 0.03 vvm and the pH was set at 7.1 by aeration of CO_2 gas.

During the entire course of the cultivation, a sample was taken every day before feeding and the samples were measured for cell concentration and viability using a cell counter. The samples were centrifuged at 190 xg at room temperature for 5 minutes and the supernatant was stored at -80°C for measurement of offline analytics, mainly, glucose, lactate, glutamine and glutamate. At the end of the cultivation, the protein concentration was quantified using Protein A column in High-performance liquid chromatography (HPLC).

The nomenclature used to represent the data is FBT2_XXX where the FBT2 stands for the fed-batch of the process at the institute (TCI) followed by the cultivation number, these are also enlisted in appendix section 5.1. The following segment would describe the data in detail.

3.1 Comparison of different scales of bioreactor

The physical characterization was centred around the parameters that serve as important factors for cultivation and scale-up. As a prerequisite for robust process transfer and comparison, the dimensions of the bioreactor were geometrically similar and the comparison is enlisted in Table 4.

The geometrical comparison of the different scales of bioreactor provides an insight into the robust scale-up possibility of the bioprocess. The bioreactors used for the project with scales ranging from 0.015 mL to 50 L are compared in the section.

The geometric relations can be described by the ratio of vessel height to vessel diameter (H/D) and impeller diameter to vessel diameter (d_R/D). The reactors are also considered geometrically similar if the ratio of the vessel diameter is in the same range as the ratio of the cube root of the volumes. An overview of the dimensions and the comparison are given in Table 4

Table 4: Geometric comparison of the different scales of bioreactor

	AMBR 15		AMBR 250	BDCU 2L	Cplus 10 L	STR 50
Total Volume [L]	0.026		0.36	3	15	68
Maximum working volume [L]	0.015		0.25	2	10	50
Minimum working volume [L]	0.010		0.10	1	3.5	12.5
Height (H) [mm]	63		120	242	565	666
Diameter (D) [mm]	28.1	14.8	60	130	185	370
Ratio H/D [m:m]	2.2: 1	4.3 : 1	2: 1	1.8 : 1	3 :1	1.8 :1
Ratio $D/V^{1/3}$ [m/m]	0.12	0.006	0.1	0.1	0.09	0.1
Impeller Diameter (d_r) [mm]	11.4		26	54	78	143
Ratio d_r/D [m:m]	0.41 :1	0.77 :1	0.42:1	0.42 :1	0.42 : 1	0.39 : 1

Note: Calculation of ratio is based on information provided by vendor

Amongst the different bioreactors, AMBR 15 was dissimilar to the other bioreactors due to its cubic structure. This lead to two data points for the diameter; one for the width and the other for the length (as represented in Table 4). For the comparison of the ratios, the length (28.1mm) is considered.

The height to diameter ratio of the vessels is comparable in AMBR 15, AMBR 250, BDCU 2L and STR 50 with 2:1 in all the scales. The height is 3 times the diameter of the 10L vessel (Cplus 10L) and this is essentially a microbial bioreactor. The change in the height to diameter ratio in the 10L reactor has a significant influence on the growth of the cells in the system. They can be used for mammalian cultivations, however, the results might vary depending on the cell line and process conditions. The impact of higher initial DO was observed and a shift in the cell growth phase was studied. This was discussed in Section 3.2.3.

The other parameter used for comparison is the ratio of diameter of the vessel and the impeller. The impeller diameter is 0.42 times the diameter of the vessel in all the 5 scales of bioreactor used here. The ratio of the diameter of the vessel and the cube root of the volume is also comparable in the different scales of bioreactor and the value is set to be within the range of 0.09 to 0.12 [m/m].

In the following section the tip speed, Reynolds number and Newton's number is compared for the different scale.

Table 5: Mixing characteristic in different scales of bioreactor

	AMBR 15	AMBR 250	BDCU 2L	Cplus 10 L	STR 50
Speed Range [rpm]	300 - 1500[Ŧ]	440 - 1320[Ŧ]	20 - 2000[Ŧ]	20 - 1500[Ŧ]	20 - 240[Ŧ]
Stirrer speed used [rpm]	1050	860	433	294	161
Tip Speed range (utip)	0.18 – 0.89	0.6 – 1.79	0.06 – 5.65	0.08 – 6.12	0.14 -1.79
Tip speed used (utip)	0.63	1.17	1.22	1.2	1.21
Reynolds number (Re) [-]	4.54×10^2	1.94×10^3	4.2×10^3	5.96×10^3	10.97×10^3
Power number (Np) [-]	2.15[Ŧ]	1.34[Ŧ]	1.3[Ŧ]	1.3[Ŧ]	1.3[Ŧ]
Np/V [1/L]	143.3	5.36	0.65	0.13	0.026

[Ŧ]: Data from vendor

The Reynolds number is dependent upon the agitation rate, impeller diameter and kinematic viscosity (equation 12). The ideal condition for the cultivation is when the system is in the turbulent zone (Re $>10^3$). From the measured data, it can be concluded that the ideal condition for mixing was provided to the bioreactors, except for the AMBR 15 which is in the transitional zone. The reproducibility of the results in AMBR 15 in spite of it being in the transitional zone has been shown in this work.

The tip speed was calculated using the equation 15.

$$u_{tip} = \pi \cdot d_r \cdot n \quad (15)$$

Where

U_{tip} : Tip speed [rotation · m /sec]

n : Stirrer speed used [rotation per second]

d_r : Impeller Diameter [m]

The range of the tip speed varies for the different scales of the reactor, especially for AMBR 15, however the agitation rate for the different bioreactors is set up based on the constant tip speed within the range of 1.17 to 1.25 rotation metre per second. Along with maintaining the tip speed, the aeration is kept constant at 0.03 vvm for the scale up studies.

Taking all the geometric and mixing characteristics into account, the bioreactors are qualified as similar and comparable to each other, with an exception of 10L bioreactor and AMBR 15. Even with mixing characteristic dissimilarity in the two bioreactors, the cultivation is carried out in these bioreactors to study the influence on cell growth and protein production. The fundamental prerequisite for process transfer between the remaining cultivation platforms is fulfilled. The results of which are discussed in the following section.

The different scales of bioreactor used for the project ranged from a working volume of 15 mL to 50 L and shown different geometries. In a production process, it is desired to have a high mAb concentration and this is directly correlated to the growth of the viable cells during the course of the cultivation. In order to test the scalability of the reactors, the factors considered were the viable cell concentration and the final monoclonal antibody concentration.

The first parameter focused up on was the viable cell concentration (VCC). An overview of the VCC over the entire duration of the cultivation in the different types of bioreactors is depicted in Figure 8.

The process was in the batch phase for the first three days (indicated by the grey area). The fed batch started after 72 hours and the cultivation was fed with the feed medium (FMA and FMB) every 24 hours thereafter.

The growth pattern in different scales follows a similar trend. The cells reach a maximum cell density on day 7 or day 8 and were transitioned to the death phase by day 11. There was an exception to the growth pattern in the 10 L bioreactor. The viable cell density trend in AMBR 15 bioreactor is similar to the other scales despite the changes in geometry and flow.

The lag phase in the 10 L reactor was relatively longer and the maximum viable cell concentration was reached on day 10. This was attributed to the dissimilarity in the geometric dimensions of Cplus 10L (10 L stainless steel bioreactor) when compared to the other systems. However, the end cell concentration was the highest in the 10 L bioreactor. Death phase was not observed in the Cplus 10L as the cells reached their maximum capacity at the end of the duration of the cultivation. Another irregularity in the growth pattern was observed in the AMBR 250, where the VCC was lower than the other batches. The cells follow the trend until day 6 after which due to technical disturbance, the cells were under stress due to unavailability of oxygen for 12 hours. This caused the early onset of death phase in the cells.

To understand this further, a “golden batch” is created using the average data of cell growth (VCC) from all the cultivations represented in the study during the course of this project. It is indicated in orange in Figure 8. It is a standard value against which the cultivation is compared to indicate the success of the transfer of the process.

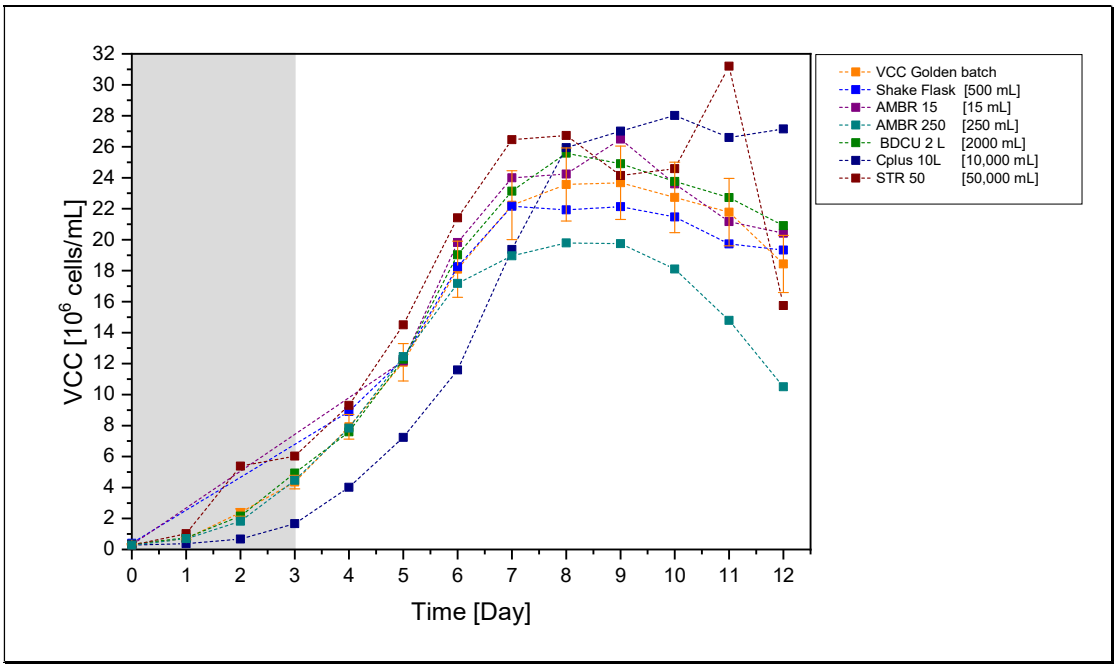


Figure 8: Comparison of viable cell concentration (VCC) in different scales of bioreactor

The viable cell concentration was one of the most critical indicator for the success of the transfer as any disturbance to the bioreactor or the process parameters would directly affect the health of the cells in these bioreactors. As per the protocol, for successful transfer of the process, the maximum VCC would have to be in the range of 17- 26 x 10^6 cells/mL and from Figure 8 it can be concluded that the cultivation was successfully transferred to the different scales of bioreactor in terms of the VCC.

The other important parameter considered for the successful transfer of the process was the final monoclonal antibody concentration. Figure 9 indicates the accumulation of the mAb during the entire course of the cultivation in the different scales. The orange line indicates the golden batch, i.e., the average protein concentration of all the cultivations.

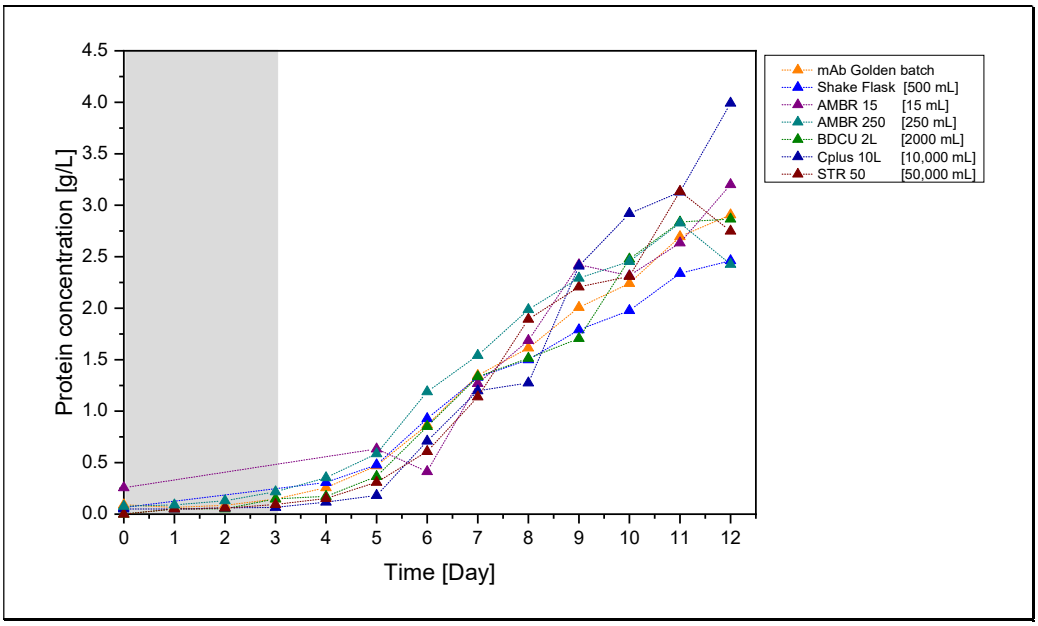


Figure 9: Comparison of final protein concentration in different scales of bioreactor

The protein concentration in the different bioreactors ranged from 2.4 to 4 g/L. The maximum concentration of 4 g/L was recorded in the 10 L reactor and the lowest of 2.4 g/L in the AMBR 250. These results correspond to the cell concentration during the cultivation, thereby indicating a direct correlation between the cell concentration and protein production.

3.1.1 Summary of scaling of process

On comparison of the different scales of bioreactor, similarity of geometric structure and mixing characterization between the different scales was evident. A vessel diameter ratio of 2:1 was maintained in all except the stainless steel bioreactor (3:1 ratio). The change in the height to diameter ratio in the 10 L reactor plays a significant influence on the growth of the cells in the system. Especially during the lag phase,` the growth of the cells was reduced but overall the highest cell density was reached in this bioreactor.

The other condition compared was the mixing characteristic within the bioreactors. On calculation of the Reynolds number for each reactor, the dissimilarity of AMBR 15 from the other scale of bioreactor was observed. An ideal condition for a cultivation is when the system is in the turbulent zone, however, AMBR 15 was measured to be in the transitional zone. Despite being in transitional zone, the cell growth was comparable to other scales of bioreactor, suggesting the difference in turbulent and transitional zone in the system did not play a notable role in the cell growth or protein production.

The comparison of different scales of bioreactor showed differences in geometry and mixing, but the cell growth and monoclonal antibody production was comparable, indicating a direct correlation between the cell concentration and protein production.

3.2 Transfer of the process

The transfer of process between different scales of bioreactor is discussed in the following section. The process transfer starts with shake flask, which is one of the most standard bioreactor in process development and is transferred to glass, stainless steel and disposable bioreactor up to the 50 L scale. This is followed by scale-down of the process to the AMBR system and this is compared to the production scale bioreactors.

3.2.1 500 mL shake flask

Shake flasks have been extensively used for the generation of biomass and seed culture. Both without baffles and with baffles shake flasks are used for different purpose during the testing within shake flasks.

For the pre-culture, shake flask with no baffles was used, however for the main cultivation, the baffled shake flasks were used to ensure sufficient oxygen transfer for the entire duration of cultivation.

500 mL shake flask with a maximum working volume of 150 mL was used for the cultivations. Shake flask triplicates were used to study the transfer of the process.

The average offline data of the first set of triplicate shake flasks used to test the transfer of the process to 500 mL shake flask was depicted inFigure 10. The highest concentration of the viable cells reached during the cultivation was 23.18 $x10^6$ cells/mL on day 8with the maximum monoclonal antibody (mAb) concentration at the end of the cultivation of 2.76 g/L.

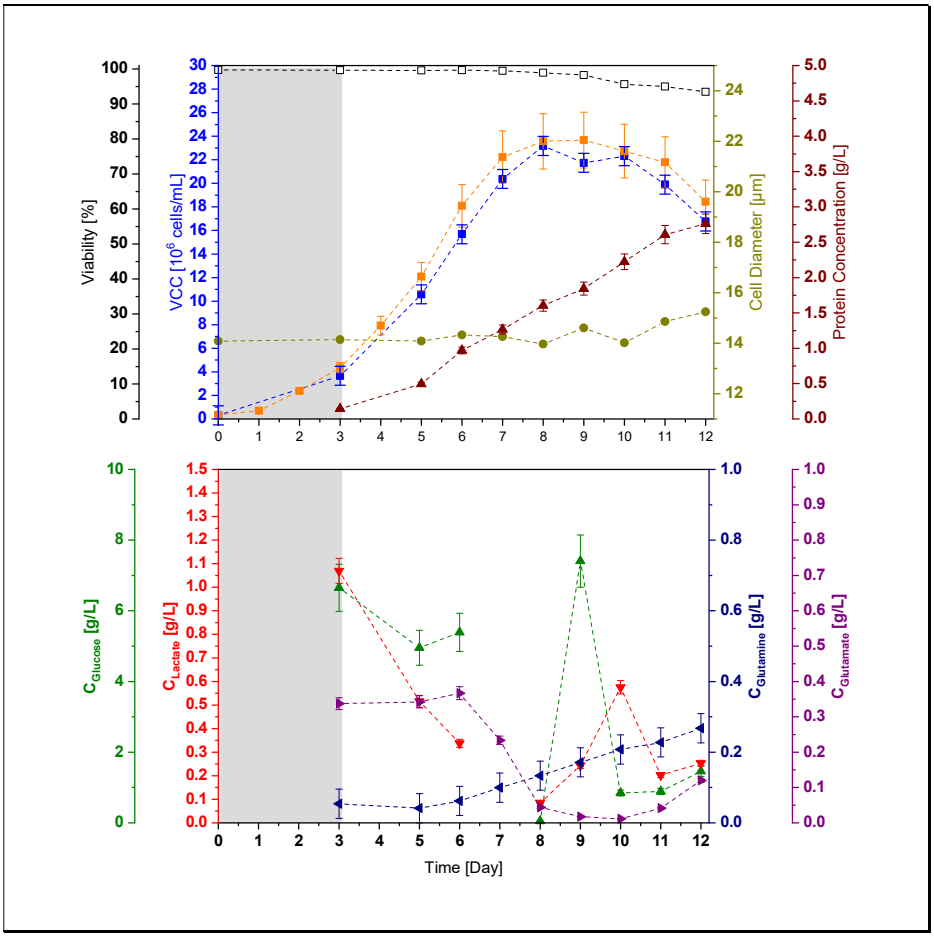


Figure 10: Offline analytics measured during cultivation in 500 mL shake flask (FBT2_008). Shown is the viability (black), VCC (blue), VCC golden batch (orange), cell diameter (olive), protein concentration (maroon) and concentration of glucose (green), lactate (red), glutamine (navy blue), glutamate (purple)

The sample values of the first two days were missing in order to reduce the stress caused by the removal of the shake flask from the incubator. On day 3, a high concentration of lactate was observed with a value of 1.1 g/L. The lactate was consumed day 3 onwards and the reduction of the concentration could be observed on day 5. The accumulation could be limited using a controlled environment.

The glucose concentration on day 3 was measured to be 6.5 g/L, indicating no substrate limitation during the batch phase, however, a limitation of glucose can be observed on day 7 and day 8, this was due to inaccurate measurement of the sample by the glucose analyser (YSI 2900). The fault in measurement resulted in an overshoot of the glucose concentration on day 9 as high amount of glucose was supplemented to all the bioreactor system on day 8 in order to maintain a concentration of 5 g/L of glucose. The glucose and glutamate

limitation on day 8 also initiated the accumulation of lactate and the trend continued until day 10 after which there was a switch in the metabolism resulting in lactate consumption. These factors had a negative effect on the cell growth and the resulting growth pattern was below the golden batch. The cells, however, grew within the acceptable range of VCC as per the golden batch data.

Along with glucose and lactate, glutamine and glutamate were also measured using the biochemistry analyser to understand the impact of it on the cell growth and protein production.

The production medium powder used for the main cultivation does not contain glutamine and this was added to the final medium product at the beginning of the cultivation. Glutamate on the other hand was available in the feed medium and was fed to the system regularly day 3 onwards.

By the end of the batch phase, the glutamine added to the medium was consumed. However, the concentration of glutamine in the system increased day 5 onwards. Glutamine is being produced by the conversion of glutamate using glutamine synthetase. The quantity of glutamate also increased on day 10; this was attributed to the death and lysis of the cells and disposal of the metabolites into the supernatant.

Following the establishment of the process in the shake flasks and attaining a cell growth within the range of the golden batch, the scale up of the production system is carried out.

3.2.2 2 L Glass bioreactor (BDCU 2L)

The next step was the scale up of the process from a simple 500 ml shake flask to an advanced bioreactor in terms of process control and monitoring.

For an industrial process, scaling up of the process is vital for the production of the desired product in a larger scale. To test the first steps in the scale up, the process was transferred from shake flasks to the 2 L bioreactor (Figure 11). This was also done due the capability of these bioreactors to continuously monitor and control the process. Two cultivations are discussed in this section, namely, FBT2_014 and FBT2_018.

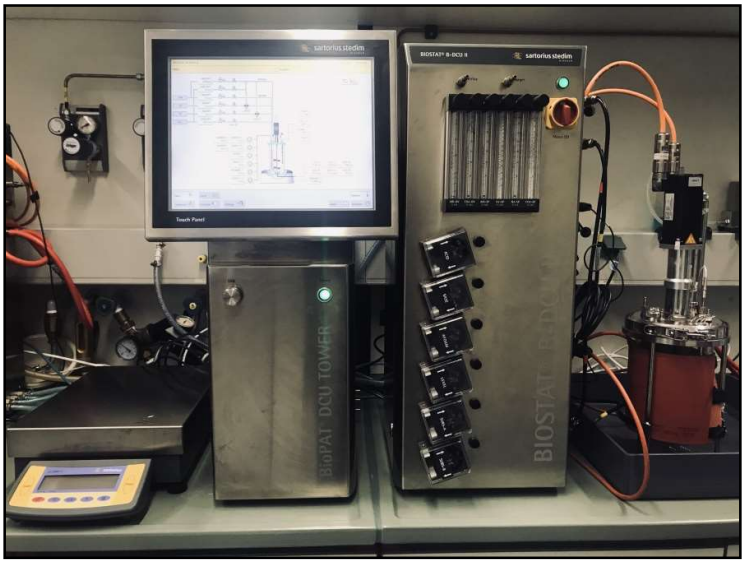


Figure 11: 2 L glass bioreactor set up

The first cultivation (FBT2_014) was represented in Figure 12. The cells reached the stationary phase on day 6 with the maximum cell concentration of 17.57 x 10^6 cells/mL on day 11 and final protein concentration of 1.68 g/L. The final product concentration is below the acceptable range of 2.5 g/L to 5g/L.

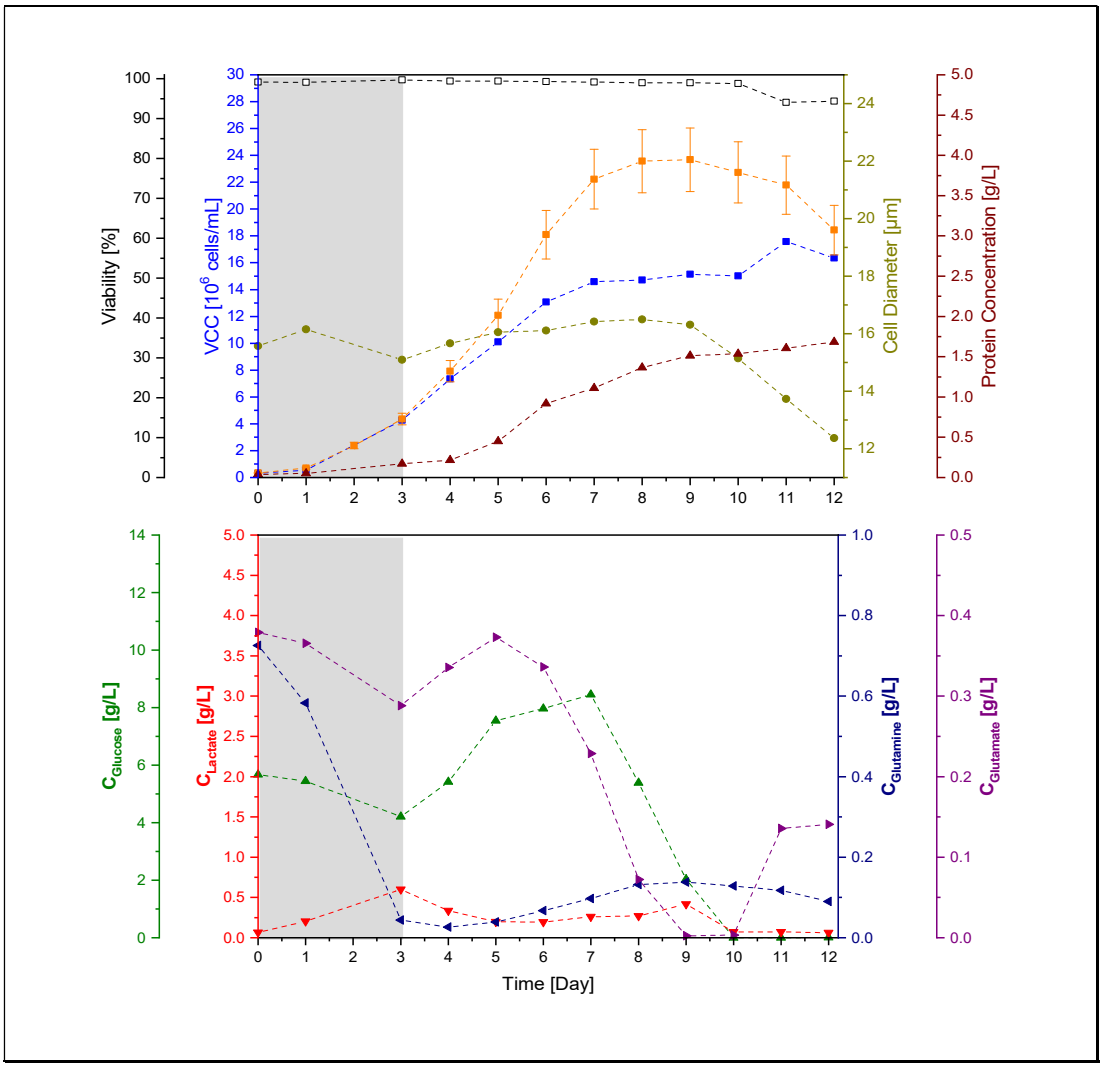


Figure 12: Offline analytics measured during cultivation in 2 L glass bioreactor (FBT2_014). Shown is the viability (black), VCC (blue), VCC golden batch (orange), cell diameter (olive), protein concentration (Maroon) and concentration of glucose (green), lactate (red), glutamine (navy blue), glutamate (purple)

The VCC pattern in the bioreactor followed the growth pattern of the golden batch and was comparable to the different scale of bioreactors until day 4. The cells transitioned into stationary phase on day 7 with a cell count of 14.59 x 10^6 cells/mL.

The viability did not indicate any fault during the first process phase and decreased from 99.2% to 94.4 % by the end of the cultivation. This was however within the expected range of the cell. The cell diameter was also considered to test the physiological conditions of the cells. There was a decrease in the cell size day 9 onwards indicating the cells were under physiological stress there on.This stress could be attributed to the change in the feeding pattern during the cultivation as a deviation from the expected growth pattern was clearly

visible from day 6. The feeding of both the feed media was paused in order to avoid over feeding of nutrients including glucose, as the value was above 8 g/L. The feeding was temporarily suspended to control accumulation of glucose. This was done to avoid increasing the osmolality of the medium and thereby hindering the uptake of nutrients by the cells.

A change in metabolism is observed in the culture with the cells shifting to both glucose and lactate consumption on day 3 and lactate was consumed until the feeding stopped. After the discontinuation of feeding, the lactate concentration increased and reached a maximum until day 9. The cells switched the metabolism to an efficient source of energy, glucose. The accumulation of lactate continued until the glutamine was consumed. On day 9, the metabolism switched to the consumption of lactate instead of glucose and glutamate. However, this was an ineffective source of energy and the cells proceeded to die.

Since the feeding was discontinued after day 6, glutamate concentration in the medium was depleted by day 9. Glutamate has a significant influence on the production of the protein and the insufficient amount resulted in low quantity of monoclonal antibody (depicted in Figure 12).

The overall cell growth and protein production deviated from the path of the golden batch. The concentration of substrate was sufficiently present in the medium until day 10, after which glucose limitation can be observed. Apart from the substrate, gassing within the bioreactor was investigated to study the root cause of the deviation earlier in the process in detail.

Gassing in the bioreactor controls the dissolved oxygen (DO) and pH of the cultivation by a mixture of nitrogen, oxygen and air for DO control and CO_2 for pH control. The influence of these gases on the cell growth was studied in detail in this segment.

The main factor considered was the aeration with carbon dioxide for the maintenance of pH in the system. Figure 13 depicts the pH value along with the VCC and carbon dioxide flow rate during the entire course of the cultivation. The pH was mainly controlled using carbon dioxide gas. During feed media FMB addition, the pH value increased above the set point and CO_2 gassing increased to control the pH to the set point. The flow rate of the CO_2

required to maintain the pH increased after the second day of feeding (day 4) and the trend continued until the day the feed media was stopped, i.e. after day 6.

The maximum gassing of CO_2 set in the control unit of the 2 L bioreactor was 25 ccm. On day 9 the CO_2 aeration gradually increased to the maximum range although the pH was nearly constant at set point of 7.10.The cells began to die after day 9 and this resulted in fluctuated requirement of gas to maintain the pH in the bioreactor.

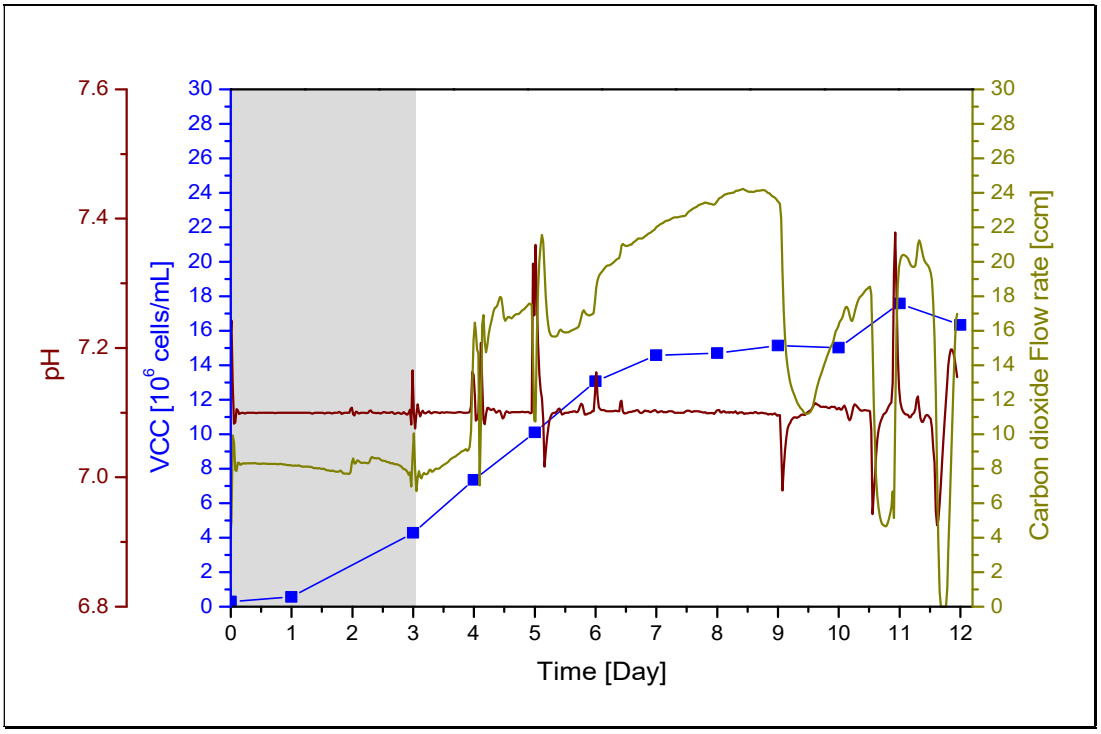


Figure 13:Aeration controlling pH within the 2L glass bioreactor (FBT2_014). Shown as pH(maroon), VCC (blue) and carbon dioxide flow rate (olive)

The overall concentration of carbon dioxide gas required to maintain a pH of 7.1 was high and this negatively influenced the cells. On day 9, when the cells began to descend into the death phase, the aeration of CO_2 required by the system decreased and fluctuated until the end of cultivation. It was hypothesized that the fluctuated and high concentration of CO_2 has a negative influence on the cell growth and eventually protein production.

The line supplying gas to the bioreactor was upgraded and a single gas line was designated to supply CO_2 to the bioreactor. The next cultivation took place using the new gas supply chain.

Figure 14 depicts the growth of the cells during the cultivation in the same 2 L reactor but with modified gas supply.

The maximum VCC achieved during the process was 25.59 x 10^6 cells/mL on day 8. The VCC was comparable to the golden batch and the successful transfer of process in terms of viable cell concentration to 2 L bioreactor was achieved. The end viability was 96.8% with a final product concentration of 2.9 g/L.

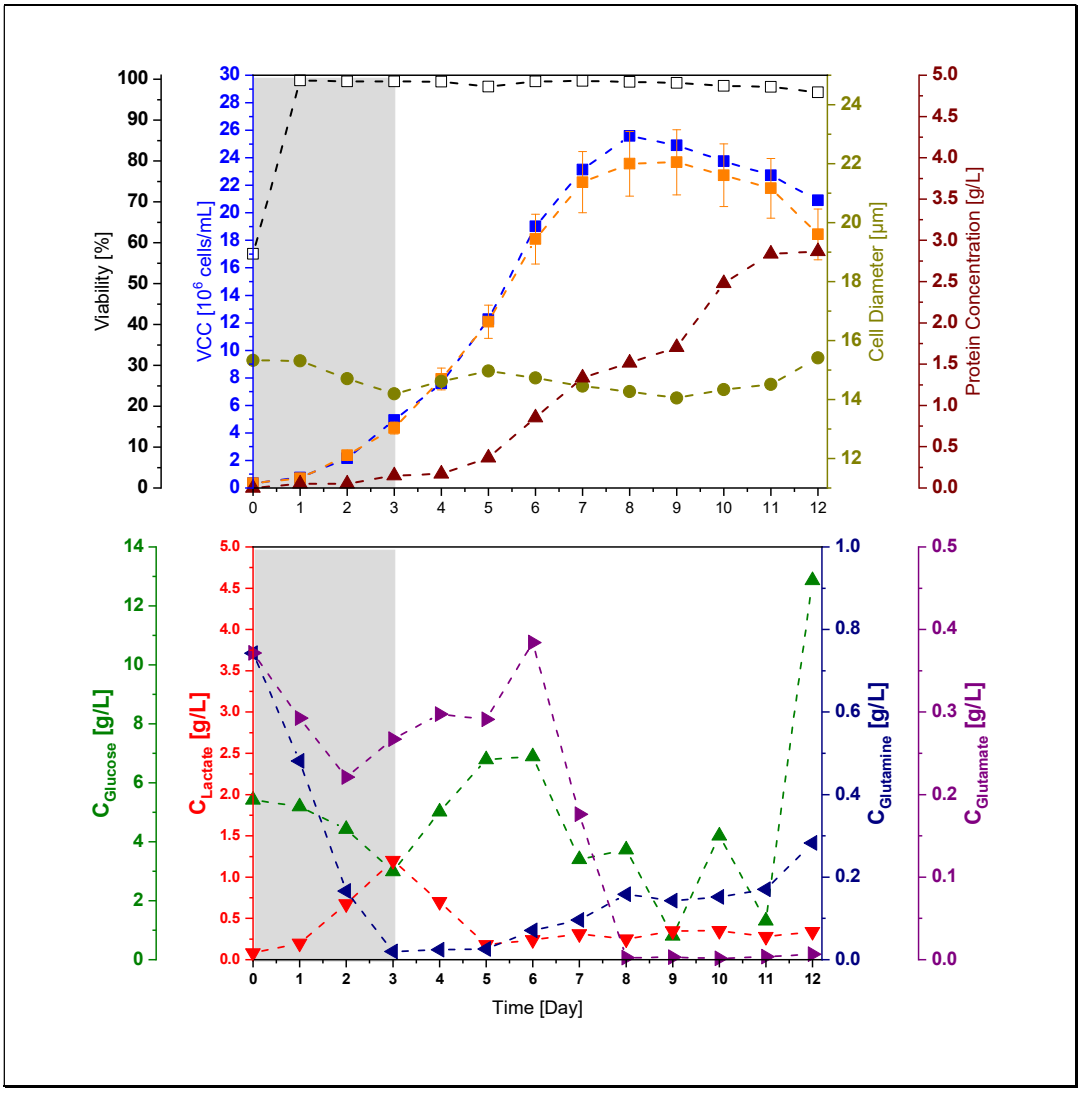


Figure 14: Offline analytics measured during cultivation in 2 L glass bioreactor (FBT2_018). Shown as the viability (black), VCC (blue), VCC golden batch (orange), cell diameter (olive), protein concentration (maroon) and concentration of glucose (green), lactate (red), glutamine (navy blue), glutamate (purple)

On studying the offline analytics of the cultivation, it can be observed that glucose was sufficiently present in the medium till the end of the cultivation and it ranged from 0.8 g/L to

7.0 g/L except on day 12. The lactate production in the culture was measured between 0 g/L to 1.5 g/L and it accumulated until the limited concentration of glutamine was available. Lactate was consumed by the culture until day 5 and remained constantly low throughout the cultivation correlated to the gradual increase of glutamine concentration in the medium.

Glutamate was fed to the culture daily during the fed batch via feed media and was completely consumed by day 8. It was metabolised constantly until the end of cultivation. Glutamine was completely metabolised by day 3; an increase in the concentration was observed from day 5 to day 8. The concentration of glutamine remained constant day 8 onwards and increased only on day 12. The protein produced also increased the production between day 9 and day 11, indicating the use of glutamate for protein production, after which it was stagnant again.

The aeration of carbon dioxide was also studied and compared to FBT2_014 cultivation. During the cultivation, pH fluctuation was observed and this was due to the addition of the feed and antifoam. The pH was regulated at 7.1 and the amount of CO_2 fed to the system was monitored and recorded using MFCS software.

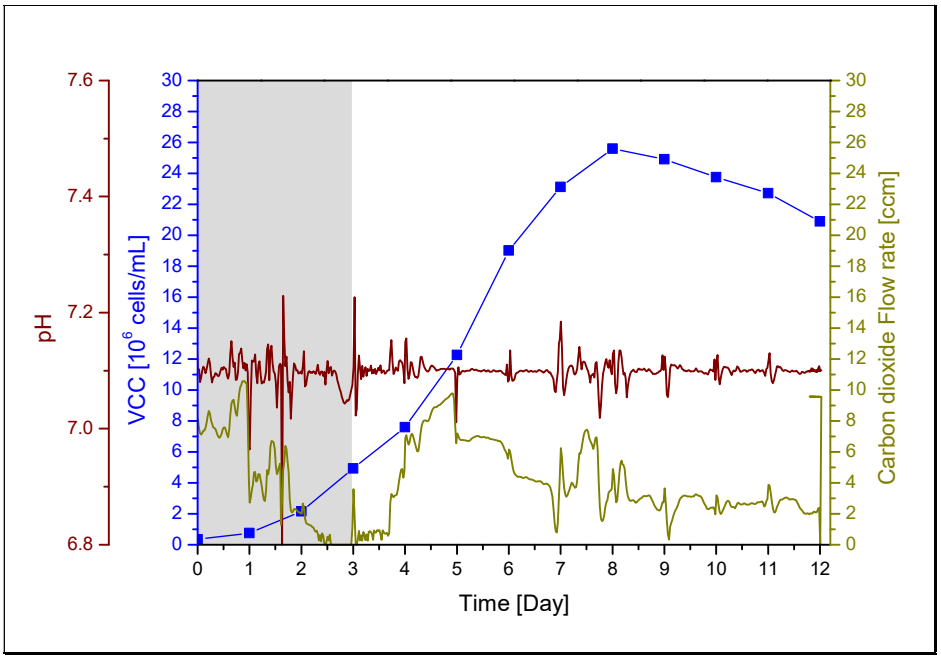


Figure 15: Aeration controlling pH within the 2L glass bioreactor (FBT2_018). Shown as pH(maroon), VCC (blue) and carbon dioxide flow rate (olive)

Figure 15 depicts the pH control in the 2 L bioreactor during FBT2_018 cultivation. The cell

growth was comparable to the golden batch and the main difference between the two cultivations (FBT2_014 and FBT2_018) was the amount of CO_2 supplied to the reactors to maintain the pH . The maximum flow rate of carbon dioxide used to maintain the pH was 10 ccm; where as in FBT2_014, the system required 2.5 times higher amount of CO_2 (25 ccm). This was also caused due to insufficient amount supplied due to faulty gas supply to the bioreactor.

Another major difference was the addition of feed medium to the bioreactor. Since the cells followed the expected growth pattern, the feeding continued as planned due to the demand of nutrients by the cells.

Therefore, it can be concluded that both, the amount of CO_2 sparged into the bioreactor and feed medium supply had a direct influence on the cell growth. This is studied in detail in AMBR 15 in Section3.3 and 3.7.

The VCC in FBT2_018 was within the acceptable range of the golden batch and the successful transfer of process in terms of viable cell concentration to 2 L bioreactor was achieved. The advantage of using the bioreactor for better regulation and control compared to shake flask aided in understanding the process further. The next step was to increase the maximum working volume to 10 L.

3.2.3 10 L stainless steel bioreactor (Cplus 10L)

The process transfer to a stainless steel bioreactor opens the horizon for process monitoring by inline sensors like spectroscopy. The 10 L stainless steel bioreactor used for the research is shown in Figure 16. The bioreactor is equipped with six ingold ports which can be used for various purpose during the cultivation such as use of spectrometers or creating a by-pass system within the bioreactor which can be further used for continuous cultivation.

Figure 16: 10 L stainless steel bioreactor used for scalability test

The 10 L bioreactor used for the project was advantageous with respect to sampling during the cultivation. An autosampler was incorporated into one of the six ingold ports in the bioreactor and a sample was extracted every 4 hours and placed in disposable chilled tubes. This provided a much more comprehensive data about the path followed by the cells during the 12 days of cultivation (Figure 18).

Figure 17 displayed the offline analytics for a single sample collected immediately before the feeding of the bioreactor.

The maximum VCC reached on day 10 with a value of 28.01 x 10^6 cells/mL with a final titre concentration of 4 g/L. This was the highest titre value achieved in the 5 different scales of bioreactor. However, during the cultivation, a longer lag phase of the cells was observed. The cells reached a stationary phase on day 8 (Figure 17).

During the execution of the cultivation, the tip speed and aeration was maintained constant at a value of 1.2 m/s and 0.03 vvm respectively. The longer lag phase was speculated to be caused due to high oxygen concentration during the batch phase and difference in k_La due to different height to diameter ratio when compared to the other scales of bioreactor. Along with cell count and viability, the cell diameter was also studied to inspect any negative effect of these changes on the cell growth apart from long lag phase. The cell diameter was within the range of 14 to 16 µm, indicating no physiological changes due to stress occurred on the cells during the cultivation.

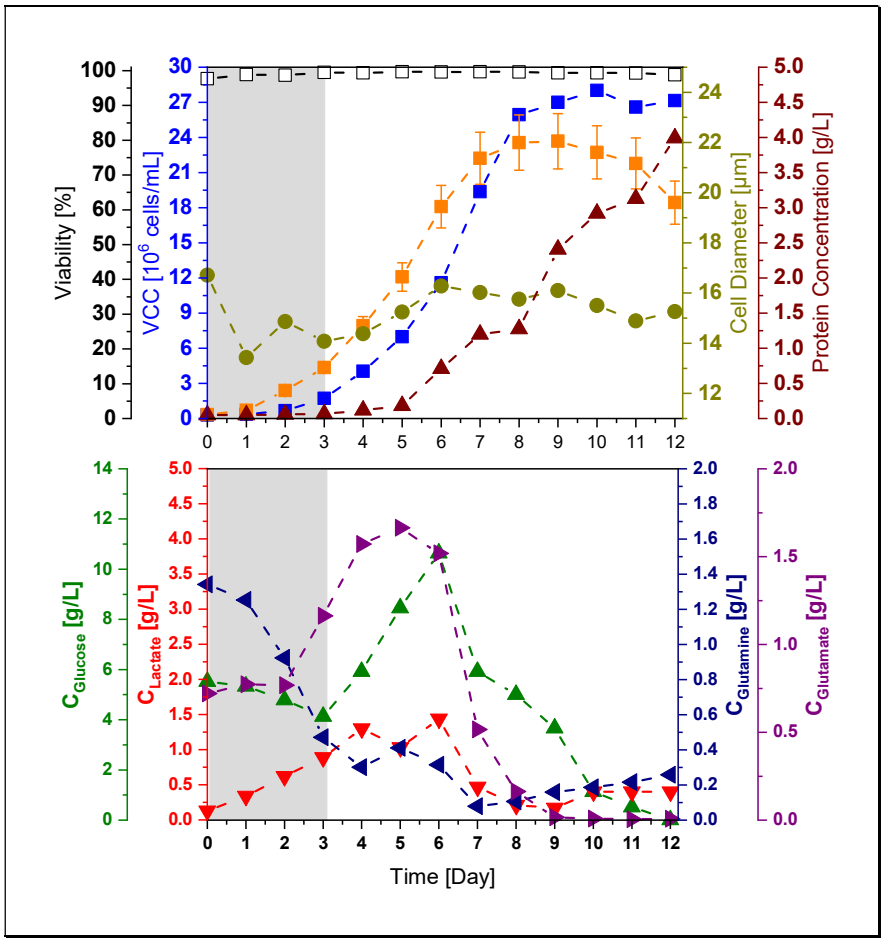


Figure 17 : Offline analytics measured during cultivation in 10 L Cplus (FBT2_015). Shown as the viability (black), VCC (blue), VCC golden batch (orange), cell diameter (olive), protein concentration (maroon) and concentration of glucose (green), lactate (red), glutamine (navy blue), glutamate (purple)

The glucose concentration reached a maximum value of 10 g/L on day 6 due to slow growth rate, along with periodic feeding of feed medium (containing 75 g/L of glucose) according to the protocol. The amount of lactate increased to 1.5 g/L on day 6. The cell concentration increased to twice the amount between day 6 (11.59 x 10^6 cells/mL) and day 7 (19.37 x 10^6 cells/mL). During this time, the consumption of glucose, lactate and glutamate drastically increased. The cells reached the stationary phase there after (on day 8) and the production of monoclonal antibody intensified.

The glutamate consumption followed a similar pattern to glucose, i.e., the amount of glutamate accumulated until day 5 followed by metabolism by the culture. Similar to other scales of bioreactor the protein production intensified after the consumption of glutamate.

Despite presence of sufficient amount of carbon and nitrogen source in the culture medium till the end of cultivation, the cell growth deviated from the expected growth pattern. This, along with longer lag phase led to investigation of the gassing within the bioreactor system. It was observed that the batch phase was carried out in a DO condition higher than the set point. A reference of DO control set point can be visualised in Figure 45.

The system was unable to maintain a starting DO of 60 % due to inability to maintain gas mixture of nitrogen and air in the beginning of the cultivation. The reactor system was supplied with only air and oxygen resulting in high DO at the beginning of the cultivation.

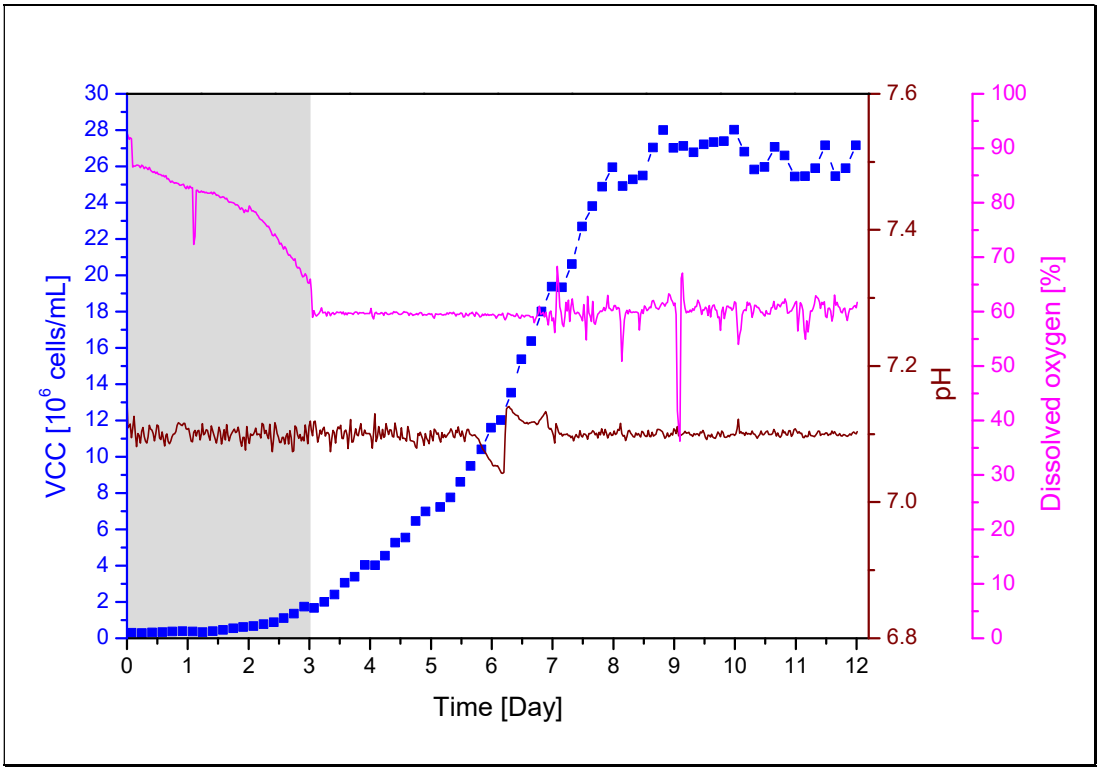


Figure 18: Process parameter in 10 L Cplus bioreactor (FBT2_015). Shown is VCC (blue), pH (maroon) and dissolved oxygen concentration (pink)

DO and pH were measured during the entire course of the experiment and the system maintained the process parameters within the desired range (Figure 18). There was a drop in DO on day 1 and day 9 due to addition of larger amount of antifoam (10 mL). The drop and subsequent increase in the pH on day 6 was due to pH recalibration of the system.

Figure 18 also shows the viable cell concentration of the samples collected with the help of autosampler. During the batch phase the cell growth was negligible and they exponentially

grew during the fed batch. It was hypothesized that the longer lag phase was due to combination of high DO percentage at the beginning of the cultivation and change in k_La caused by dissimilar geometric ratio of the bioreactor when compared to the other reactors. The influence of high DO on the cell growth was further studied after scale down in the AMBR system and is described in section3.4.

The 10 L bioreactor fulfilled the process transfer criteria of VCC ranging from 17 – 26 x 10^6 cells/mL and mAb concentration ranging from 2.5 g/L to 5 g/L. The process was successfully scaled up to 10 L stainless steel bioreactors enabling a better process monitoring by higher frequency of offline sampling.

3.2.4 50 L Single-use stirred tank reactor

Production of monoclonal antibodies takes place in single-use bioreactors due to a wide range of advantages of the system. The single-use stirred tank bioreactor of maximum working volume of 50 L (Figure 19) was used to demonstrate the scalability of the process in the production scale.

Figure 19 : Single use stirred tank bioreactor with a maximum working volume of 50 L

In the STR 50, the maximum VCC reached on day 11 with a value of 31.2 x 10^6 cells/mL and a viability of 98.8 %. The cells switched from exponential to stationary phase on day 7 with a VCC of 26.45 x 10^6 cells/mL (Figure 21). The stationary phase was short lived due to

insufficient supply of oxygen for 12 hours between day 7 and day 8 (Figure 20). The influence of which was observed in later state of the cultivation. The cells began to die by the end of day 10 and thereby reducing the requirement of oxygen to maintain DO at 60 %. By the end of the cultivation air and nitrogen were used to maintain the dissolved oxygen concentration in the bioreactor.

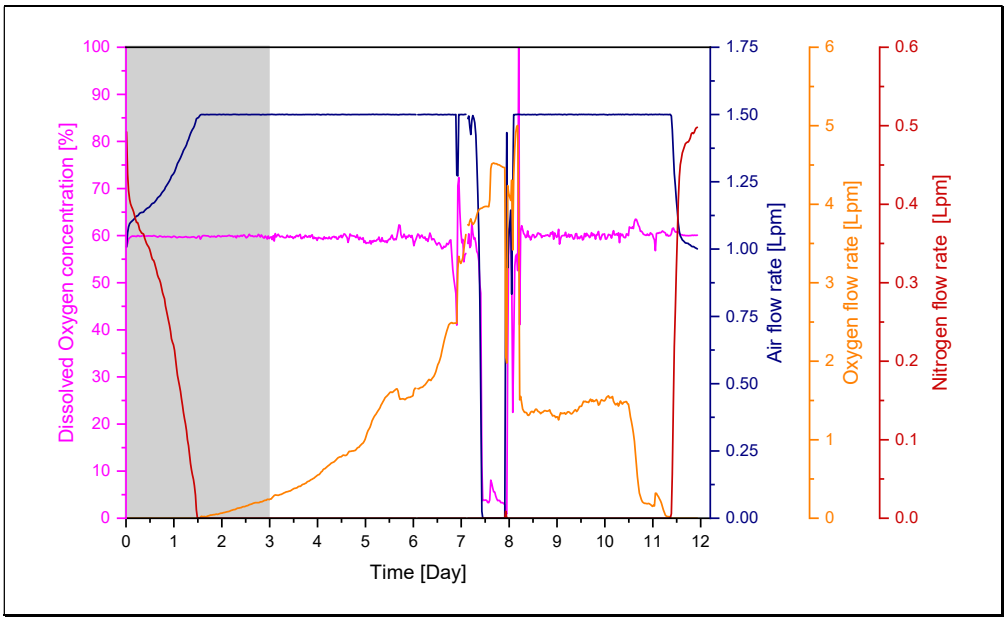


Figure 20: Aeration controlling DO in STR 50. Shown is air flow rate(navy blue), oxygen flow rate (orange), nitrogen flow rate (red) and dissolved oxygen concentration (pink)

The offline samples were measured to determine the protein concentration and it was observed that the titre increased gradually until day 10 and there was a steep increase in the concentration on day 11 followed by a decrease in the concentration. The sample was measured thrice in order to test if this was an outlier. However, the results were reproducible. This piqued the interest of determining the reason for the consumption. It was speculated that at the final cell viability of 45 %, most of the cells were lysed and the proteases were released into the medium and this resulted in the breakdown of the protein produced.

Figure 21 depicts the offline analytics measured during the cultivation and the cell growth pattern was comparable to the golden batch but were 15 % higher than the expected values.

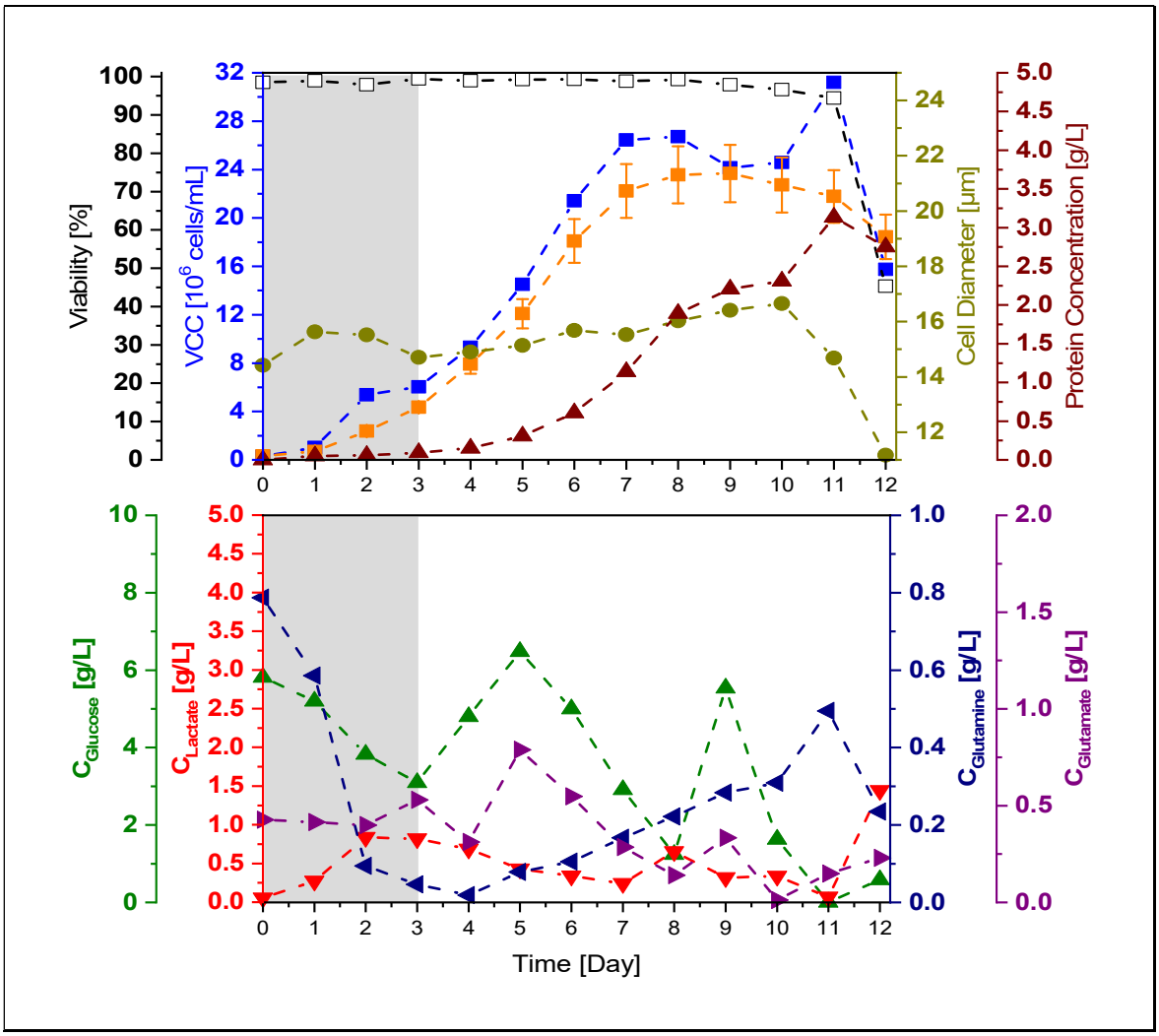


Figure 21 : Offline analytics measured during cultivation in 50 L single-use bioreactor (FBT2_020). Shown is the viability (black), VCC (blue), VCC golden batch (orange), cell diameter (olive), protein concentration (maroon) and concentration of glucose (green), lactate (red), glutamine (navy blue), glutamate (purple)

Approximately 2.79 g/L of glucose was consumed during the batch phase and the amount gradually increased until day 5. Glucose limitation occurred on day11 with a concentration of 0.06 g/L in the medium. The glucose supplemented on day 10 was completely metabolised by day 11. The increase in the growth to 31.2 x 10^6 cells/mL on day 11 supported this reading.

Lactate production and consumption was negligible at the larger scale. The maximum lactate concentration was measured on day 2 and day 3 with an amount of 0.8 g/L. The concentration increased again on day 12 to 1.45 g/L due to stress and release of the component from the lysed cells.

Glutamine and glutamate followed a similar pattern as observed in the shake flask and 2 L glass bioreactor with glutamine being completely metabolised by day 4 followed by accumulation of the component in the supernatant. The cells produced approximately 0.5 g/L of glutamine by day 11 and consumed 0.25 g/L by day 12.This corresponds to the increase in the concentration of lactate.

The criteria for successful transfer and scale-up of the process ranging from 2 L to 50 L bioreactor was reached. This indicated the robustness of the process and similarity in scalability of the cultivation.

3.2.5 250 mL Microbioreactor (AMBR 250)

The next step was to scale-down the process to single-use microbioreactors which would in turn be used to study the impact of various factors on the cell growth and protein production.

The first automated microbioreactor used of the study was the AMBR 250. These are geometrically similar to the conventional bioreactor.

Figure 22: Microbioreactor with maximum working volume of 250 mL (AMBR 250)

The pH and DO within the bioreactor was maintained using only a mixture of pure oxygen, air (for DO regulation) and carbon dioxide (for pH regulation). Nitrogen was not used by the reactor system. All the sections of the single-use bioreactor was filled with the production medium, antifoam and feeds under the clean bench and placed in the control unit. The reactor was inoculated under the sterile environment of a laminar air flow bench and samples were manually taken every 24 hours before feeding.

Figure 23 represents the data generated from the offline samples. The maximum VCC was reached on day 8 with a value of 19.8 x 10^6 cells/mL with a viability of 98.8 %. The viability at the end of cultivation decreased drastically to 58.4%. This was caused due to stress caused by abrupt discontinuation of gassing in the bioreactor system between day 7 and 8. The cells started to shrink as well and the end diameter value was measured to be 12.42 µm by the cell counter system. A similar decrease in cell diameter pattern was observed to STR 50 where the oxygen supply abruptly stopped for 12 hours between day 7 and day 8.

The titre increased to 3.05 g/L until day 11 and decreased to 2.90 g/L on day 12. The pattern was similar to the one observed in STR 50. The decrease on the final day was speculated to be due to the release of proteases and breakdown of the desired protein.

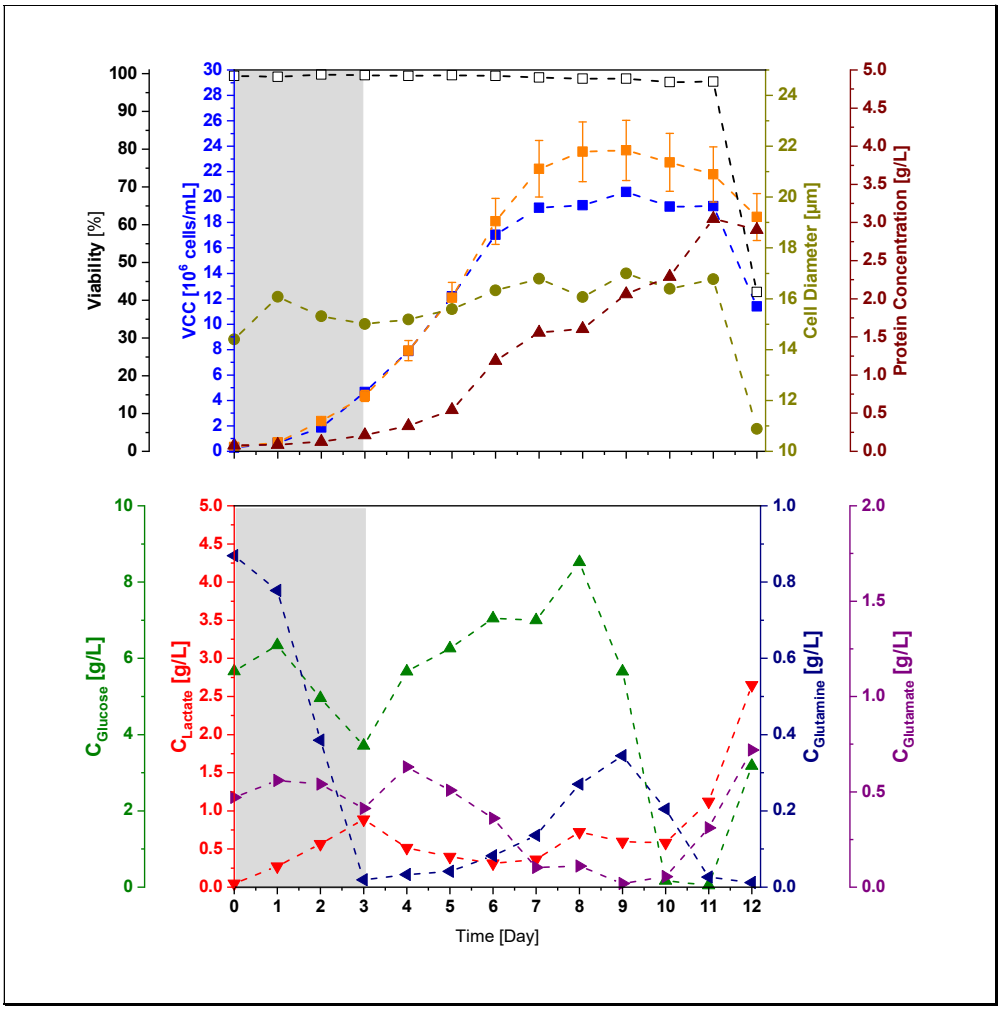


Figure 23: Offline analytics measured during cultivation in 250 mL microbioreactor. Shown is the viability (black), VCC (blue), VCC golden batch (orange), cell diameter (olive), protein concentration (maroon) and concentration of glucose (green), lactate (red), glutamine (navy blue), glutamate (purple)

The glucose was consumed until day 3 from 6 g/l to 4 g/L. The value increased on day 1 and

this was considered an outlier caused due to error in biochemistry analyzer. The concentration increased until day 8 as the FMA was being fed every day but the amount of glucose was not completely being metabolised. Lactate was accumulated to 1.0 g/L until end of batch phase and consumption of glutamine. It was further consumed by the cells on the following days. The concentration increased again after day 7 until the end of cultivation to 2.65 g/L due to rapid consumption of glutamine by the end of the cultivation.

In Figure 23, the viable cell was compared to the golden batch and the concentration was within the golden batch until day 6. The concentration deviated from the projected trajectory due to fault of the disposable bioreactor supplied by the vendor. The gassing stopped in the system due to “over pressure”. This was rectified by removing the vessel from the culture station and cleaning the station. The vessel was placed back into the station but DO and pH sensors were damaged during this time. The value of the DO remained zero in spite of continuous supply of oxygen.

The aeration within the reactor was studied in detail to decipher the reason for the change in the growth pattern. Figure 24depicted the gassing of air and oxygen for the control of DO. The gassing and DO decreases to 0 % on day 7. The gassing was stopped due to over pressure and the single-use bioreactor was removed from the station, cleaned and placed back to AMBR 250 system. The gassing resumed for a day and was disrupted due to “over pressure” again. Visual inspection of the filter showed no sign of clogging, suggesting vessel was defective.

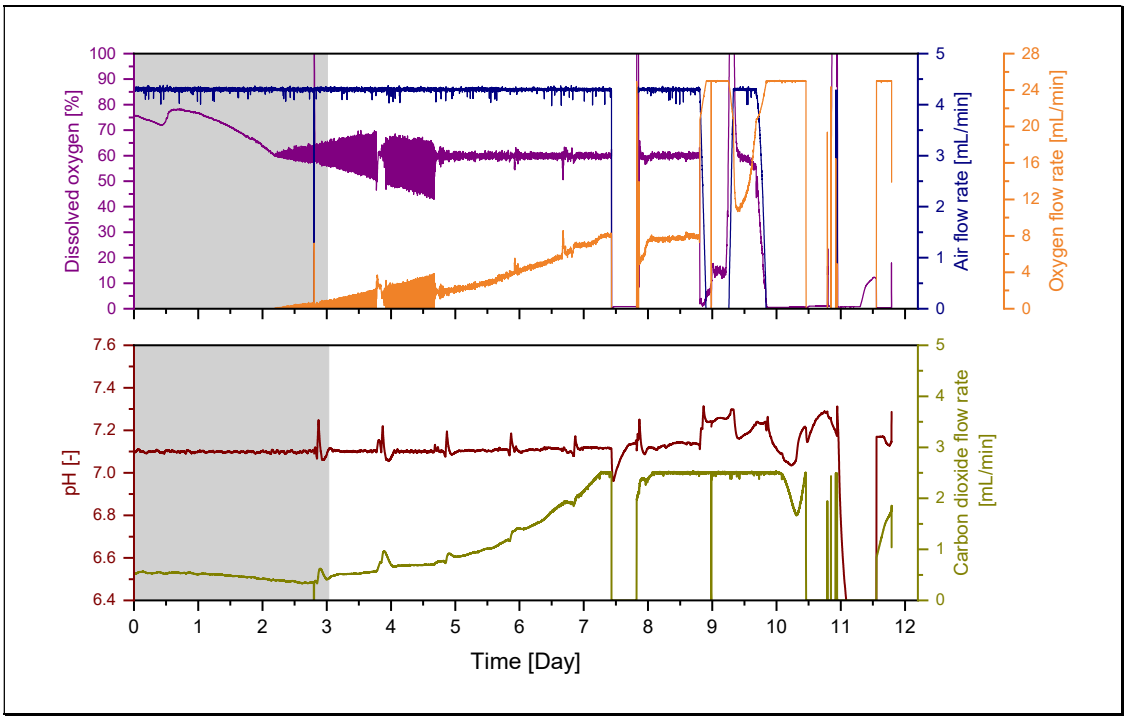


Figure 24: Aeration control in AMBR 250

The offline sample from day 8 were also observed under the microscope to eliminate the suspicion of contamination. The cells were damaged but no contamination was observed.

The cells have the potential to reach the golden batch with a new batch of vessels. The end product in AMBR 250 was measured to be 2.9 g/L which was attributed to the low cell concentration in the bioreactor. This can be rectified and tested further but the final product concentration provide sufficient evidence of the success of the transfer of the process.

3.2.6 15 mL Microbioreactor (AMBR 15)

The next scale of automated microbioreactor used was the AMBR 15 (as in Figure 25).

The maximum working volume of AMBR 250 is 250 mL which was 16.6 times higher than AMBR 15. However, 24 vessels of AMBR 15 makes it an ideal bioreactor system for process characterization studies, with a condition that the cell growth and metabolic activity of the cells are comparable to the large scale bioreactors.

Figure 25 : Microbioreactor (AMBR 15)

Automated micro bioreactors such as the AMBR 15 provide an advantage in form of multiple parallel cultivations with different working conditions, medium and process optimization options. Therefore, scale-down of the process is equally important. If the results were reproducible in the smaller scale and the results were within the accepted golden batch range, the small scale micro bioreactors could be used for design of experiments and optimization of the medium, inoculum, feeding strategy, process parameters to name a few.

One of the disadvantages, however, is the low volume because of which one sample was taken after the inoculation to obtain the initial VCC and continued after day 5. It was assumed that the path taken by the cells was the same as projected by the golden batch in case the results were within the golden batch range.

Figure 26 depicts the offline analytical values of the samples measured during the course of the cultivation. The maximum VCC was reached on day 9 with a value of 26.5 x 10^6 cells/mL, however, the stationary phase began on day 7 with a VCC of 23.99 x 10^6 cells/mL. The viability at the end of the cultivation was 96.6 % and the final mAb concentration was 3.2 g/L.

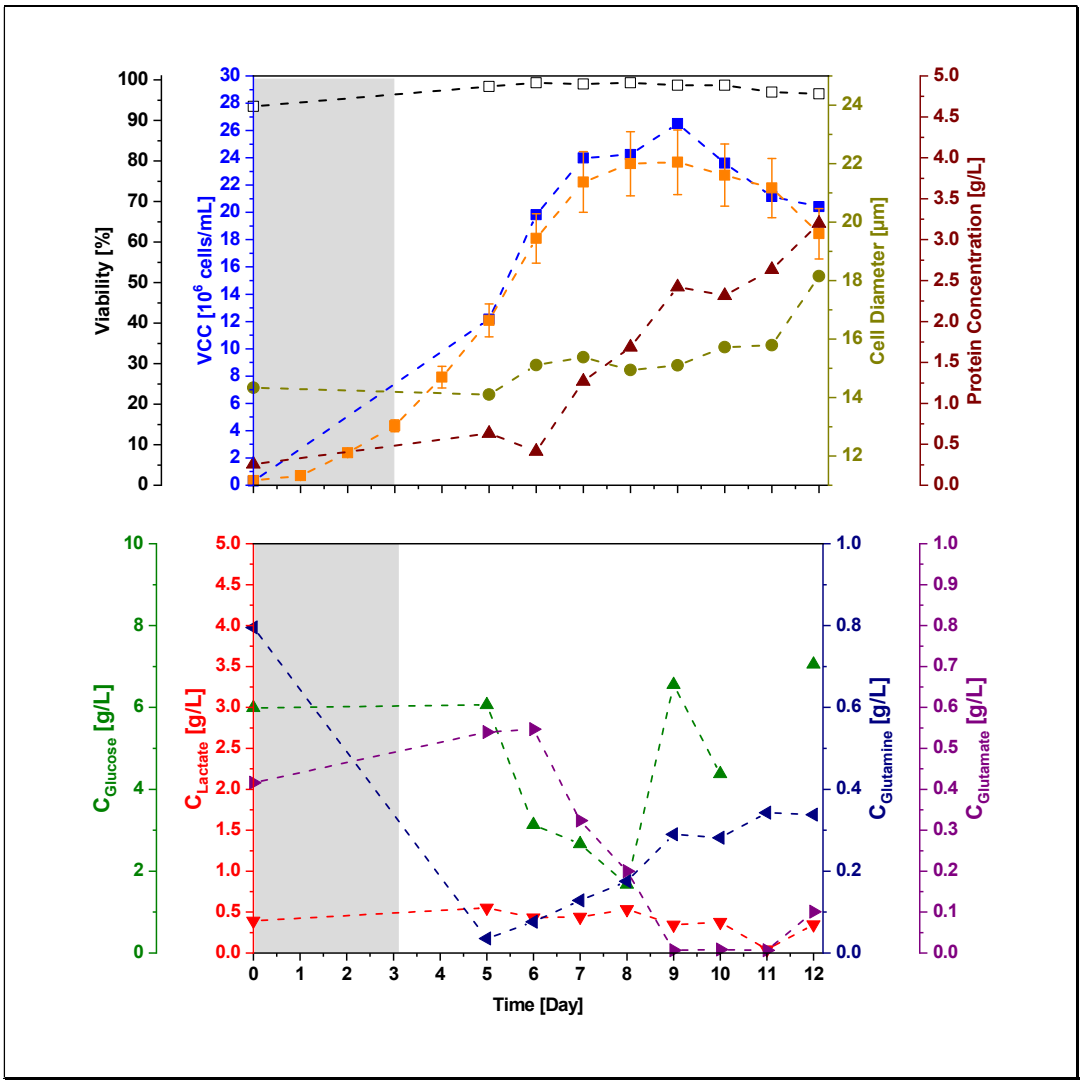


Figure 26 : Offline analytics measured during cultivation in 15 mL microbioreactor (FBT2_021). Shown is the viability (black), VCC (blue), VCC golden batch (orange), cell diameter (olive), protein concentration (maroon) and concentration of glucose (green), lactate (red), glutamine (navy blue), glutamate (purple)

The data for the first 4 days was unavailable due to lack of sampling, the shift in the metabolism to consume the lactate was not visible, however, the concentration of lactate remained below 0.5 g/L during the entire course of the cultivation.

The glucose component was sufficiently present during the entire cultivation. The value reached a peak of 6.5 g/L on day 9 due to addition of 400 g/L of glucose to the system to maintain a glucose value of 5 g/L. This was within the expected range and no glucose limitation occurred during the entire course of the cultivation.

The cell growth followed the path of the golden batch and glutamine was consumed by the cells before day 5. Glutamate supplied to the culture was metabolised by the cells for

protein production and a small amount of glutamate was used for glutamine production. The absence of glutamate in the supernatant from day 9 to day 11 indicated the complete metabolism of the component for the basic functionality of the cells. During which phase, production of protein decelerated. An increase is observed when sufficient amount of glutamate is available for the cells to consume.

From the data represented in Figure 26, it can be concluded that the cell growth and protein concentration were comparable to the large scale bioreactor and scale-down of the process to 15 mL automated microbioreactor was successful. It can be used for cell line selection and design of experiments pertaining to process and medium optimization. These were further used for studies in sections 3.3, 3.4, 3.5, 3.6 and 3.7.

3.2.7 Summary of process transfer

On comparison of the different scales of bioreactor, similarity of geometric structure and mixing characterization between the different scales was evident from Table 4 and Table 5. A height: vessel diameter ratio of 2:1 was maintained in all except the stainless steel bioreactor (3:1 ratio). The other shortcoming of the 10 L bioreactor was the lack of upgraded mass flow controller. The influence of which was evident in the section 3.2.3 , where a slower growth was observed during the batch phase. This can be further investigated by testing the influence of higher oxygen concentration on the cell.

The other condition compared was the mixing characteristic within the bioreactors. On calculation of the Reynolds number for each reactor, the dissimilarity of AMBR 15 from the other scale of bioreactor was observed. An ideal condition for a cultivation is when the system is in the turbulent zone, however, AMBR 15 was measured to be in the transitional zone. Despite being in transitional zone, the cell growth was comparable to other scales of bioreactor, suggesting the difference in turbulent and transitional zone in the system did not play a notable role in the cell growth or protein production.

The systems were also compared to each other in terms of IVCC, average cell specific productivity, maximum VCC and final mAb concentration. The summary is listed in the table below:

Table 6 : Summary of the different scales of bioreactor

	Shake flask (500 mL)	2 L BDCU	10 L Cplus	50 L SUB (STR)	AMBR 15	AMBR 250
Maximum VCC [10^6cells/mL]	23.43	25.59	28.02	31.20	28.22	20.40
IVCC [10^6 cells*day/mL]	150.23	177.46	166.22	198.79	199.72	147.61
Maximum titre [g/L]	3.00	2.87	3.99	3.13	3.54	2.90
Average cell specific productivity [pg/cell/day]	19.43	17.10	23.02	15.68	19.26	20.11

The maximum viable cell concentration ranged from 20.40 x 10^6 cells/mL to 31.20 x 10^6 cells/mL with the final product ranging from 2.75 g/L to 3.99 g/L. These values were within the acceptable range for the transfer of the process to different scales, i.e, a VCC range from 17 x 10^6 cells/mL to 26 x 10^6 cells/mL and titre range from 2.5 g/L to 5 g/L.

It can be concluded that the process was robust and was scalable in bioreactors ranging from 15 mL to 50 L. During the course of study in the different reactors various process parameters seemed to play an important role in cell growth and protein production. These process parameters were further studied to optimize the process.

Due to multiple parallel cultivation that can be conducted in AMBR 15; this bioreactor was used for the optimization of process parameters during the cultivation.

3.3 Process optimization: Influence of pH

In this segment, the influence of pH on the growth of CHO DG44 was studied using AMBR 15 microbioreactors (FBT2_016). This was conducted to measure the normal operating range within which the cell functionality would not deteriorate.

The process parameters such as DO (60%), temperature (36.8 °C) and agitation (1050 rpm) were kept constant for all the bioreactors. The only varying parameter was the pH; and the set points were maintained at 6.9, 7.1(control) and 7.3. The main attributes used for the comparison were integral viable cell concentration, cell specific productivity and final product quantity.

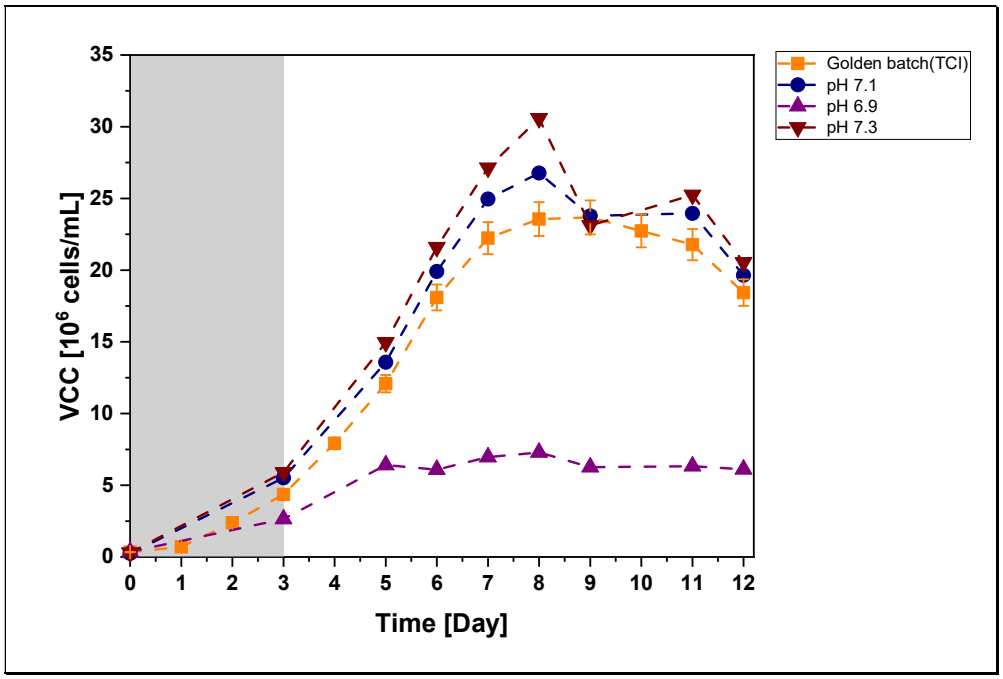


Figure 27: Viable cell concentration in the measured to test influence of pH in AMBR 15

Figure 27depicts the viable cell concentration in the bioreactor during the entire course of the cultivation. The orange line represents the average of all the reactors used for the study in this thesis and is used as a standard against which all the cultivations would be compared to with respect to the viable cell concentration; this is referred as the golden batch.

The two set of bioreactors with pH set points of 6.9 and 7.3 were compared against the control bioreactor (pH of 7.1).

To test the impact of comparatively acidic condition on the cells, the pH set point was reduced by 0.2 from the control (pH 7.1). The cell growth in the bioreactor with pH 6.9 was 3.8 times lower than the cell growth in the control bioreactor maintained at pH of 7.1, with a maximum VCC of 7.3 x 10^6 cells/mL and final product concentration of 0.93 g/L.

The influence of alkaline condition on cell growth and protein production was also tested by increasing the pH set point by 0.2 and maintaining the pH in the reactor to a set point of 7.3. A positive impact on the cells growth as observed. The cells reached a higher VCC of 30.58 x 10^6 cells/mL on day 8. A dip in the VCC was noted on day 9, this is considered an outlier. Overall, the VCC value was 1.1 times higher than the control (pH 7.1)and the final product quantity was measured to be 2.52 g/L.

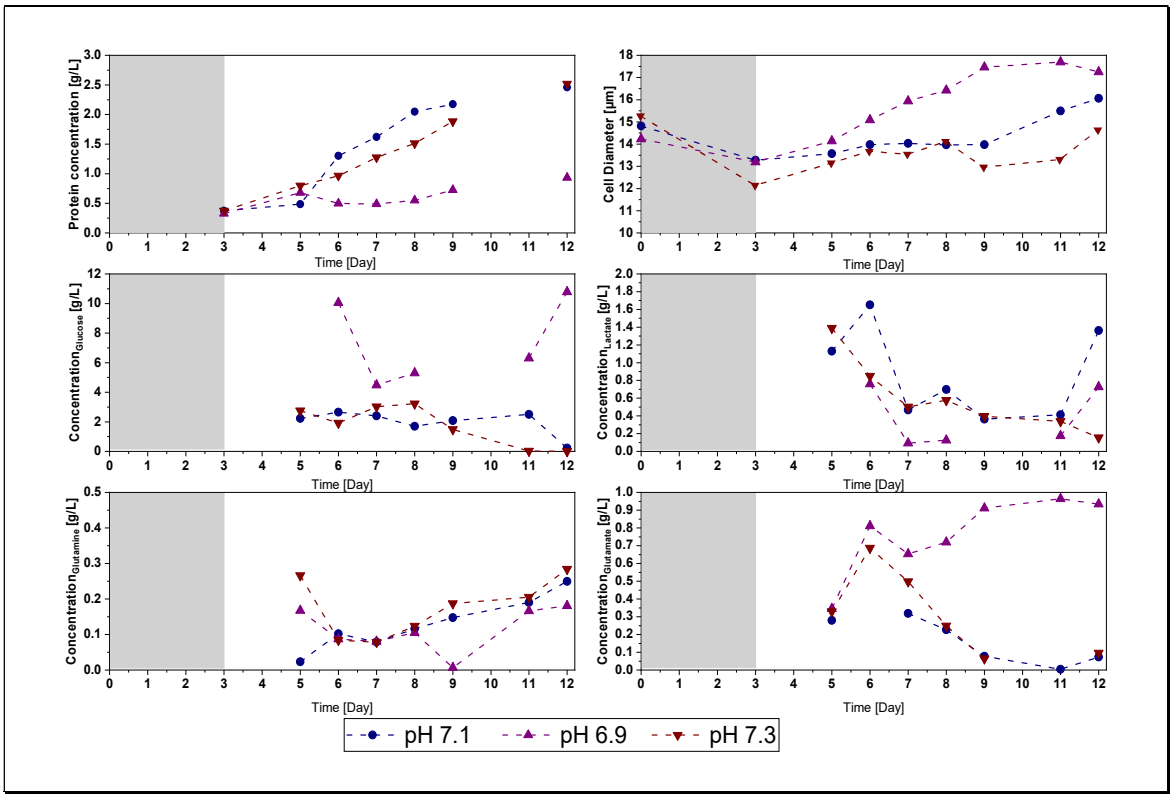


Figure 28 : Offline analytics measured to test influence of pH in AMBR 15

The decrease in pH resulted in an increase in the concentration of CO_2 added to the system to maintain the low pH. The amount of CO_2 added to the bioreactor system is shown in Figure 29.A combination of pH and CO_2 caused stress in the cells resulting in an increase in the cell diameter (Figure 28). Higher concentration of pCO_2 in the medium resulted in lower product quantity and cell proliferation.

In the bioreactor with pH set point of 6.9, the lower growth rate resulted in slower glucose metabolism and in turn 2 to 5 times lower lactate production when compared to the control (depending on the day of cultivation).

The glutamine metabolism follows a similar pattern to the other two set of bioreactors with gradual increase in the concentration by the end of the cultivation; however, glutamate was accumulated in bioreactor with pH set point of 6.9. This was attributed to low viable cells concentration in the bioreactor leading to lower uptake of glutamate for cell metabolism and protein production.

The product quantity, glucose and glutamine metabolism in the control (pH 7.1) and bioreactor with pH set point of 7.3 were comparable and it was concluded that the impact of alkaline pH was significant on cell proliferation; however, it did not have any difference on the final protein concentration.

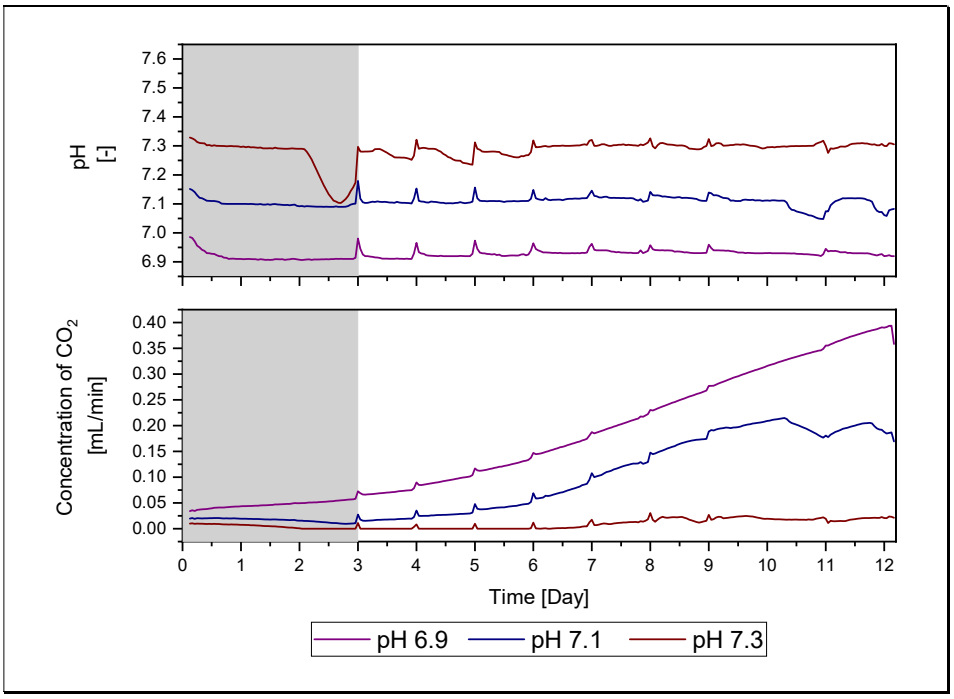


Figure 29: pH control and CO2 gassing in AMBR 15

Figure 29 depicts the pH measurement and the quantity of CO_2 added to the microbioreactors during the entire course of the cultivation. The system was able to maintain the set point throughout the cultivation.

A gradual decrease in pH was observed in bioreactor with pH set point of 7.3 between day 2 and 3 because higher pH resulted in higher carbon flux thereby increasing the lactic acid production, which in turn leads to decrease in extracellular pH. The pH increased after the start of feed due to addition of FMB which has a pH range of 11.0 to 11.4 and thereafter the system continuously maintained the pH set point till the end of the cultivation.

The flow rate of CO_2 added to the bioreactor with pH set point of 7.3 was measured to range between 0.00 to 0.05 mL/min and the bioreactor with pH 7.1 required a maximum of 0.15 mL/min of CO_2 to maintain the pH. The requirement of CO_2 to maintain the pH in the bioreactor with pH set point of 6.9 gradually increased to 0.40 mL/min. This was 2.7 times higher than the amount required for control bioreactor. The influence of high CO_2 flow rate was also seen in the 2 L bioreactor (FBT2_014) in section 3.2.2. It can be concluded that a high quantity of carbon dioxide gas has a detrimental effect on the cell proliferation and product quality.

Table 7 : Summary of output variables in the process optimization studies (pH)

Bioreactor set	IVCC [10^6 cells*day/mL]	Final titre [g/L]	Average cell specific productivity [pg/cell/day]
pH 7.1 (Control)	187.59	2.46	29.00
pH 7.3	199.77	2.52	25.51
pH 6.9	60.20	0.93	29.22

Table 7 summarizes the total IVCC, final titre and average cell specific productivity measured for the three sets of bioreactors. The bioreactors with pH 7.1 and 7.3 have comparable cell growth and final titre concentration, however, the average cell specific productivity is comparatively higher in pH 6.9 when compared to pH 7.3. This implies that the amount of cells producing the protein were comparatively active in pH 6.9.

From the overview, it can be concluded that alkaline and acidic parameters not only influence the overall metabolism but also cell growth and productivity. The set point of 7.1 is ideal for the cultivation and can be increased up to 7.3 with positive influence on cell proliferation and cell specific productivity with no negative effect on the product concentration.

3.4 Process optimization: Influence of Dissolved Oxygen (DO)

The results of the experiments during process up- and down-scaling demonstrated that dissolved oxygen was an important factor for the cell growth and product production. A decrease in cell growth and protein production was observed in STR 50 and AMBR 250 after issues with supply of oxygen.

In present study, the one factor to be varied was the dissolved oxygen concentration in the microbioreactors (FBT2_021). This was conducted to deduce the influence of oxygen on cell growth and protein production along with finding the normal operating range of the cultivation in terms of DO.

The parameters such as temperature (36.8 °C), pH (7.1) and agitation (1050 rpm) were set constant in the bioreactors. The three DO set points used for the study were 40 %, 60 % (control) and 80 %. The impact of this was measured based on its influence on cell concentration and final titre at the end of the cultivation.

Figure 30 represents the viable cell concentration of the three sets of bioreactors with set points 40 %, 60 % and 80 %. These cells are compared to the golden batch.

The cell growth was significantly better in the bioreactor with the DO set point of 60 %. The peak VCC was observed on day 8 with a value of 28..22 x 10^6 cells/mL and the final product quantity was measured to be 3.54 g/L.

Bioreactors with set point 80 % and 40 % showed a similar trend to each other with a maximum VCC of 25 x 10^6 cells/mL on day 8 in both and a final titre of 3.05 g/L for bioreactor with DO 80 % and 3.39 g/L for bioreactor with DO 40 %.

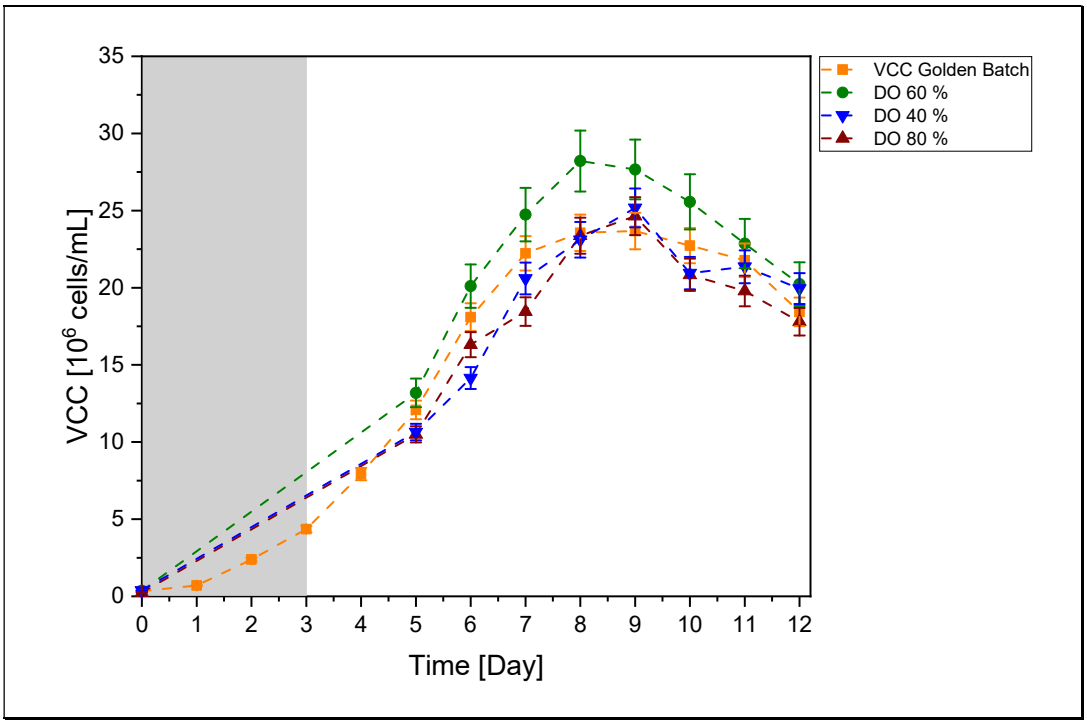


Figure 30 : Viable cell concentration in the measured to test influence of DO in AMBR 15

Figure 31 presents the offline metabolic variable along with the protein concentration and cell diameter. The product production was not influenced significantly by the different set point of DO and the metabolism of glucose and glutamine was comparative in the three sets of bioreactors. However, since the cell proliferation in the reactor with DO set point of 60% was faster than the other set points, the metabolism of glutamate was rapid in the control bioreactor, with glutamate being depleted in the bioreactor on day 9 when compared to day 11 for both bioreactors with DO 40 % and 80 %. This glutamate consumption cannot be ascribed to the product formation, as seen in Figure 31 but to the cell growth. Therefore, glutamate is used for both, cell growth and production formation in nearly equal parts.

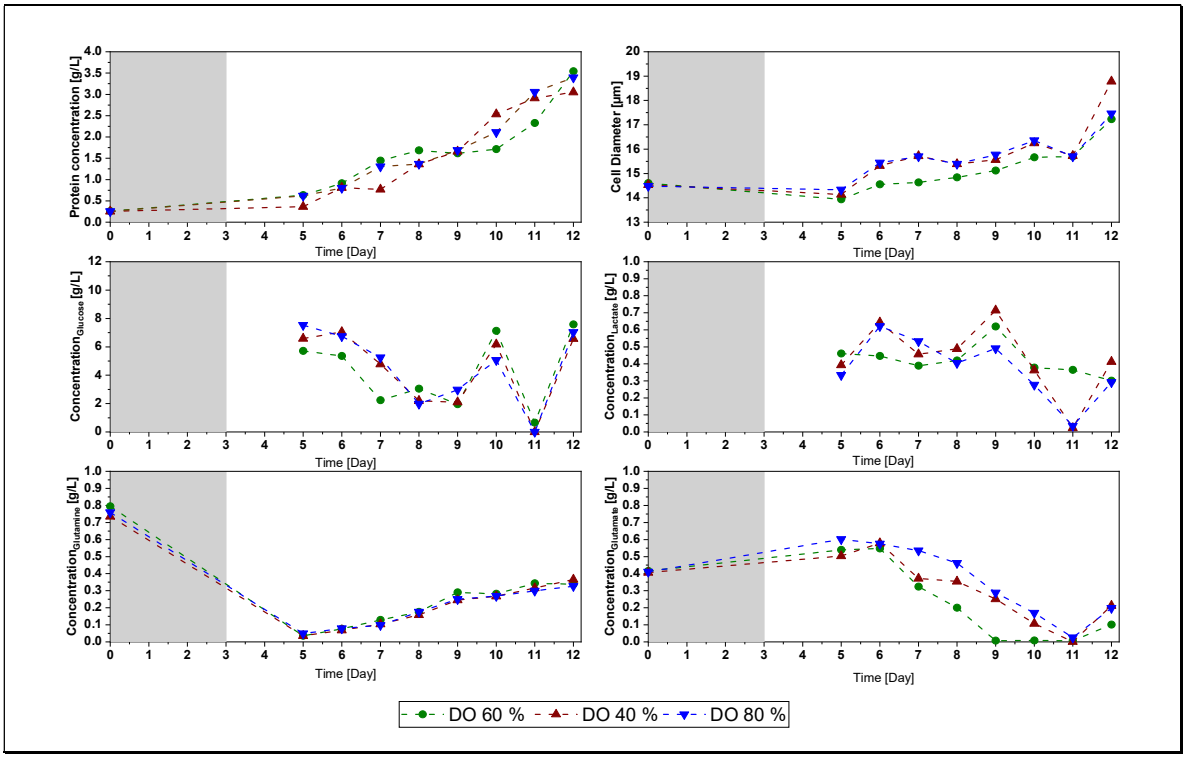


Figure 31 : Offline analytics measured to test influence of DO in AMBR 15

From this experiment, it can be suggested that the decreasing the DO set point from 60 % to 40 % would not have a negative impact on the process and it could be used while scaling up of the process. Here an oxygen limitation was not tested, but a decrease to 40 % is not critical for the process. Moreover, it could be used while scaling up of the process. One of the advantage was reduction in the requirement of pure oxygen at the production scale and thereby comparatively reducing the amount of oxygen driven free radicals which could have detrimental effect on the protein and DNA of the cells.

Table 8 : Summary of output variables in the process optimization studies (DO)

Bioreactor set	IVCC [10^6 cells*day/mL]	Final titre [g/L]	Average cell specific productivity [pg/cell/day]
DO 60 % (Control)	199.72	3.54	19.26
DO 80 %	164.41	3.05	18.11
DO 40 %	168.21	3.39	22.48

Table 8 summarizes the total IVCC, final titre and average cell specific productivity. The bioreactors with DO set point of 40 % and 80 % contained similar IVCC at the end of the cultivation but the average cell specific productivity was higher in cells cultivated at a DO of

40 %, suggesting more cells in bioreactors with set point 40 % were actively producing the desired protein. The final titre in all the three set of bioreactor ranges between 3.05 g/L to 3.54 g/L and it is concluded that the reduction in the DO set point would not have a negative effect on the cells or the product quantity. The DO for the production scale can be reduced from 60 % to 40 % to produce the same amount of protein. Also, in case of 60 % oxygen, glutamate was limited at the end of the process due to higher consumption related to higher cell growth, with less growth of the cell at 40%, this substrate can be used to produce the target protein instead.

3.5 Alteration in Production medium composition

Apart from process parameters, the production medium and feed medium was also studied in detail to improve the protein production and accelerate cell proliferation. This was studied in following section.

Glucose has been widely accepted as primary source of nutrient for promotion of cell growth and functions. It was proposed by *Newsholme et. al.* [84] to consider glutamine and glutamate as equally important components to maintain cell functionality and protein production.

The initial step to explore the impact of glucose, glutamine and glutamate on cell growth and protein production was executed by altering the start concentrations in the main cultivation medium (production medium).

The experimental design was constructed as per Table 9. For easy understanding of the results, the experimental set up would be referred to by the bioreactor number listed in Table 9.

Table 9 : Production medium design to optimize medium

Bioreactors	Glucose concentration [g/L]	Glutamine concentration [g/L]	Glutamate concentration [g/L]	Comment
Set 1	6.0	0.6	0.0	Control
Set 2	6.0	1.2	0.0	Double glutamine concentration
Set 3	12.0	0.6	0.0	Double glucose concentration
Set 4	6.0	0.0	1.2	Glutamine replaced by glutamate
Set 5	6.0	0.0	2.0	Double glutamate concentration
Set 6	6.0	0.6	1.2	A combination of glutamine and glutamate

Based on the previous studies, it was observed that glutamine was completely consumed by day 3 in the different bioreactors. An experimental design was established by altering the concentration of glucose and glutamine in one set of bioreactors and doubling the quantity of each of these components individually in another set of bioreactors to test the impact on cell growth and in turn protein production, considering that higher cell concentration would result in increased protein concentration.

The other observation from the scale-up study was the metabolism of glutamate and production of glutamine during the end of the cultivation. It was also estimated that the high protein production lead to low glutamate concentration and by increased glutamate more protein could be produced.Therefore, two concentrations of glutamate (1.2 g/L and 2 g/L) were also added to the bioreactor and the effect on the cell growth and protein production was studied.

The study was carried out in microbioreactor AMBR 15. Based on the previous section, it was confirmed that the results in small-scale microbioreactor were comparable to the large scale multi- and single-use bioreactors. It can be inferred that the tests conducted in AMBR 15 would be reproduced in larger scale.

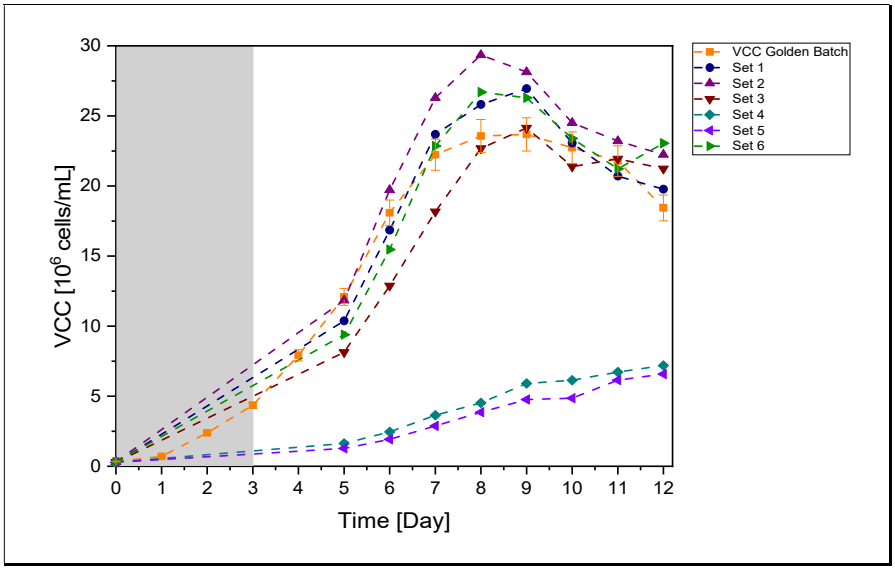


Figure 32: VCC for analysis of change in production medium

Figure 32 represents the viable cell concentration in the six different set of bioreactors. Set 1 was the control bioreactor against which the other sets were compared. The cell growth in all the sets, except set 4 and set 5 reached a comparable growth pattern to set 1 and the golden batch.

The maximum cell growth of 29.35 x 10^6 cells/mL was observed in set 2 containing double the amount of glutamine when compared to the control bioreactor. In contrast to these results, the lowest cell growth was observed in set of bioreactors containing production medium with glucose and only glutamate (no glutamine). The maximum VCC reached by these bioreactors was 7.19 x 10^6 cells/mL (set 4) and 6.58 x 10^6 cells/mL (set 5). This indicated that glutamine was essential for cell growth in the early process phase. After day 3, glutamine was metabolized and only became available by conversion from glutamate.

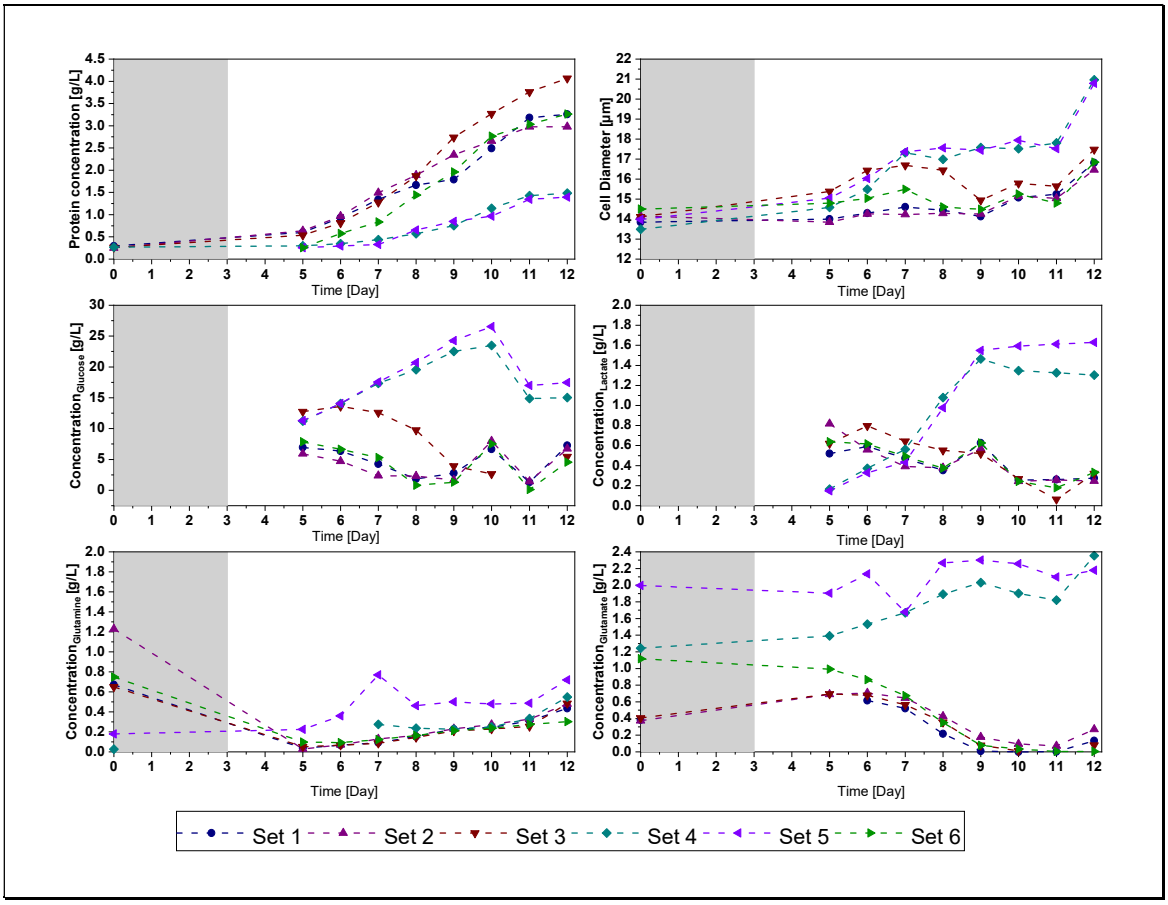


Figure 33: Offline analytics measured for analysis of change in production medium

Glucose is the primary carbon source used by the cells for cell function and acts as an oxidative fuel in most cells. It is primarily metabolized by glycolysis and TCA cycle and is used in most cells for survival, proliferation and differentiation [84]. The influence of doubling the glucose concentration in the production medium was used to study the effect specifically on cell growth.

Set 3 contained double the amount of glucose (12 g/L) in the medium. Due to high glucose

concentration, the cells growth rate was slower and this was interpolated from the data available from day 5 onwards. The cells reached a maximum cell concentration of 24.14 x 10^6 cells/mL with a final titre of 4.07 g/L. This resulted in less IVCC over the cultivation at 157 x10^6 cells*day/mL with a higher average cell specific productivity of 25.87 pg/cell/day compared to the control. The glucose consumption gradually increased day 7 onwards, indicating the high metabolism of cells during the stationary phase which also corresponded to the increase in the productivity but not in the cell growth.

The other factor studied was impact of glutamine. Glutamine is an important precursor for peptide and protein synthesis, purine and pyrimidine synthesis and in turn nucleic acid and nucleotide, it also provides carbon source for oxidation [84]. Therefore, the next step was to study the impact on IVCC and final titre by doubling the glutamine concentration in the production medium. The production medium composition in **set 2** contained 6g/L of glucose and double the initial amount of glutamine, i.e., 1.2 g/L of glutamine. The cells reached a maximum VCC of 29.35 x 10^6 cells/mL compared to set 1 (control) with a value of 26.94 x 10^6 cells/mL. The high cell concentration was attributed to the high glutamine concentration. This was conducted to study whether the increase in the cell count would in turn result in high titre at the end of the cultivation. The final titre was measured to be 2.97 g/L, this was 0.29 g/L lower compared to the control bioreactor. It is hypothesized that during proliferation and expansion of cell concentration, the production of protein is reduced. Another hypothesis was that high glutamine concentration resulted in high ammonium range and this further hindered with the productivity.

Apart from glucose and glutamine, influence of glutamate was tested on the CHO DG44 cells. Glutamate can either donate its amino group for a new amino acid synthesis (transamination) or can lose the amino group via deamination to 2-oxoglutarate. Glutamate can also combine with NH_3 by the action of glutamine synthetase and produce glutamine which can be used by the cells for growth [84]. This property was tested out in **set 4 and 5** containing no glutamine and 1.2 g/L and 2.0 g/L of glutamate, respectively. The VCC was the least in these cases and it was due to unavailability of glutamine during the initial stages of cell growth. This finding is according to set 2 with high glutamine in the beginning resulting in high cell count. On studying the glutamine profile in the bioreactor, an increase in the concentration was observed in both the sets of bioreactors with the final glutamine concentration being 0.55 g/L and 0.72 g/L for set 4 and 5 respectively, suggesting the switch

in metabolism which led to production of glutamine from glutamate. The increase in glutamine concentration in the supernatant also suggested a fraction of the amino acid was used by the cells for cell proliferation which in turn results in increase in VCC by the end of the cultivation. From the setup it was concluded that the cell line used for the study required a minimum of 0.6 g/L of glutamine to carry the normal cell growth in the beginning of the growth phase.

The last set of bioreactor contained same amount of initial glucose and glutamine concentration as the control bioreactor (set 1). The only difference being the use of additional 1.2 g/L of glutamate in **set 6**. The cell growth in both set 1 and set 6 of bioreactors was comparable and followed a similar path indicating glutamate was not needed for cell growth during the lag and exponential phase of the cells. This was supported by the comparison of glutamate concentration in the different set of bioreactors. Glutamate was completely metabolized by day 9 in all the set of bioreactors (except set 4 and set 5)according to the initial concentration in the production medium. The protein production in set 6 was slower compared to set 1; however, the final concentration was the same on day 12 with a value of 3.26 g/L in both the sets. The presence of glutamate lead to a slower production in the beginning but this is compensated later on during the cultivation.

A table summarizing the integral viable cell concentration, final titre and average cell specific protein productivity is listed in Table 10.

Table 10 :Summary of output variables in altered production medium studies

Bioreactor set	IVCC [10^6 cells*day/mL]	Final titre [g/L]	Average cell specific productivity [pg/cell/day]
Set 1	178.70	3.26	18.64
Set 2	198.65	2.98	15.48
Set 3	156.90	4.07	25.87
Set 4	38.59	1.48	30.87
Set 5	32.25	1.39	40.30
Set 6	165.67	3.18	20.11

The IVCC for the sets containing glutamine ranged from 157 x10^6 cells*day/mL to 199 x10^6 cells*day/mL. The bioreactors with only glucose and glutamate in the production medium had an end IVCC of 32 to 39 x10^6 cells*day/mL. It can be concluded that glutamine is essential for cell growth especially in the early process state. However, the average cell specific productivity of these bioreactors was 1.5 times to 2 times higher than the control

bioreactor, suggesting the amount of cells present in these conditions actively produce more protein instead of proliferating.

The conclusion from this study is that the production medium can be optimized further by increasing the initial concentration of glutamine and glucose to range between 0.6 to 1.2 g/L and 6to 12 g/L, respectively. Glutamine can be fed to the bioreactor individually as a bolus feed to be sufficiently present in the medium for the cells during the stationary and death phase. Also, presence of glutamine is essential during the initial stages of the cultivation to ensure cell proliferation. Glutamate on the other hand is not required by the cells until the start of the stationary phase and therefore a bolus feed of glutamate can be supplemented to the culture day 7 onwards to ensure sufficient amount is available for protein production and cell sustainability.

3.6 Amino acid analysis

Apart from altering the production medium, the percentage of feed medium added to the culture can also be varied and the influence of it studied on the cell growth and protein production. Also, before altering the percentage of feed medium, the consumption of each amino acid during the cultivation would provide an insight into the metabolism of the cells during different stages of growth.

Cell culture medium is made up of defined chemicals such as carbohydrate, amino acid, vitamins, minerals, lipids and proteins such as growth factor. These are important components used for the growth and functionality of the cells. During the course of the cell growth, these elements are consumed and by-products are produced and accumulated.

In the previous section, the carbon and nitrogen source consumption were discussed in detail. The focus in this section is on the amino acid consumption. The 19 essential and non-essential amino acids were measured by Reverse Phase-High performance liquid chromatography (RP-HPLC).

In the following segment, a few of the amino acids are explained in detail depending on their importance to the cell growth and protein production.

Before dwelling into the amino acid consumption analysis, it is important to understand the amino acid composition in the protein of interest, IgG1.

Figure 34 represents the percentage of the amino acids present in the genetic structure of the protein.

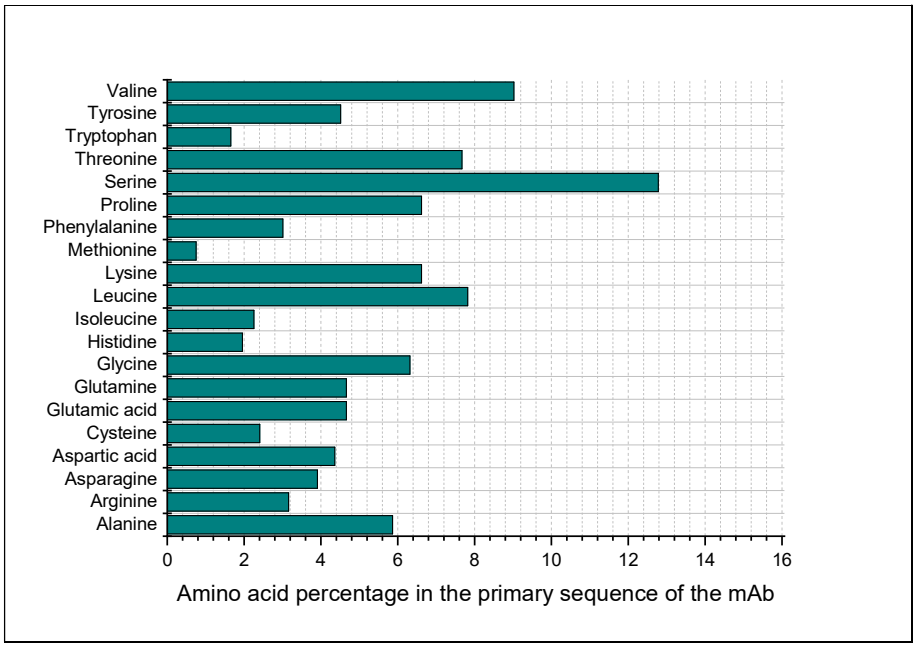


Figure 34 : Amino acid percentage in protein of interest

The most abundantly present amino acid is Serine which is 12.78 % of the total amino acids present in the genetic molecular structure of the desired protein. The other amino acids present in higher quantity are valine, threonine, leucine, proline and glycine. The amino acid present in the least amount is methionine with only 0.75 % of the total amino acid profile of the monoclonal antibody. It is vital to ensure these amino acids were sufficiently present in the medium for the cells to use and metabolise them.

To understand the medium better, the quantity of amino acid in the production and feed media (FMA and FMB) were also measured and the data was represented in Figure 35 and Figure 36.

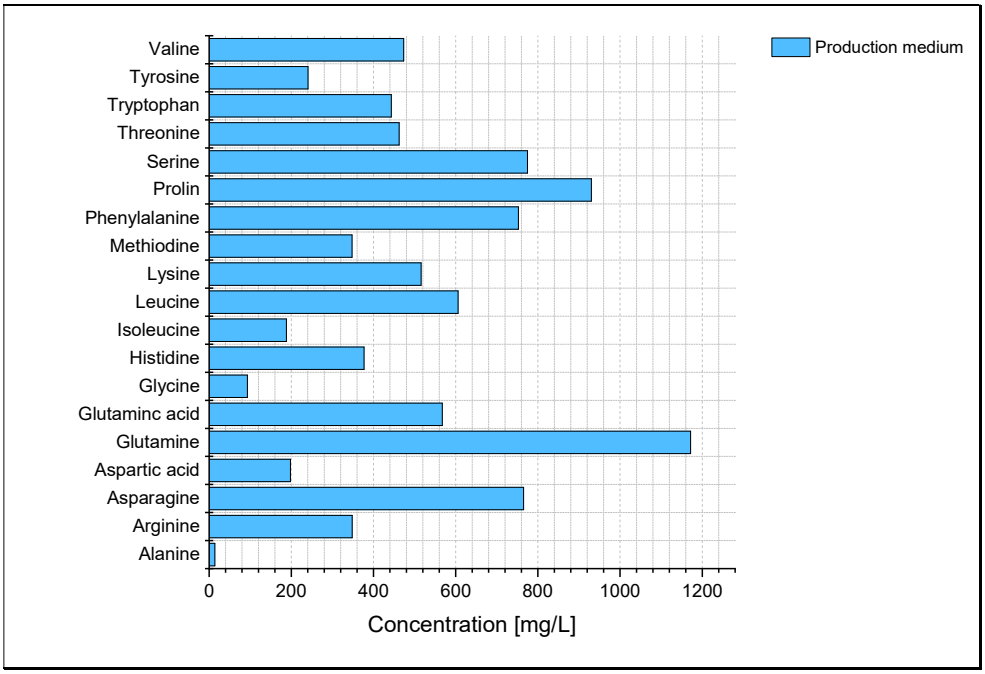


Figure 35 : Amino acid concentration in production medium

In production medium, proline, serine, asparagines and glutamine were present in higher concentration. During the preparation of the production medium, 6mM of glutamine was added to the already prepared medium, therefore was an elevated concentration inFigure 35. As discussed earlier, glutamine was used for the cell growth in the early process phase and was therefore important to be sufficiently present in the medium when the cells were inoculated into the production bioreactor.

Alanine, on the other hand, was present in the least amount in the production medium, indicating that it was not required for the initial cell growth and expansion. However, it makes up approximately 5.86% of the amino acid structure of the monoclonal antibody. The deficiency of alanine in the production medium was supplemented with the feed medium added to the production bioreactor day 3 onwards.

During the course of the cultivation, these amino acids were consumed by the cells for daily functioning, growth and protein production. Therefore, it was important to supply the depleted amino acids using the feed medium.

The concentration of amino acids supplied during the fed batch is indicated in Figure 36. The red bar graph indicated the concentration in FMA and FMB is depicted in yellow. Most of the amino acids required by the cells are supplied to the bioreactors by addition of FMA. It can also be noticed that among the essential and non-essential amino acids present in FMB, the

amount of tyrosine and tryptophan was significantly higher in the medium. However, it must be noted that the addition of FMA was ten-fold higher than FMB. Therefore, only a fraction of tyrosine and tryptophan was added to the medium during the cultivation with each feed.

Another amino acid significantly present in the monoclonal antibody was threonine. However, only a small amount of the amino acid is present in both FMA and FMB but is sufficiently present in the production medium. Based on amino acid concentration in the supernatant represented in Figure 37, it was evident that threonine was metabolised by the cells for the production of the protein and was not used for cell proliferation.

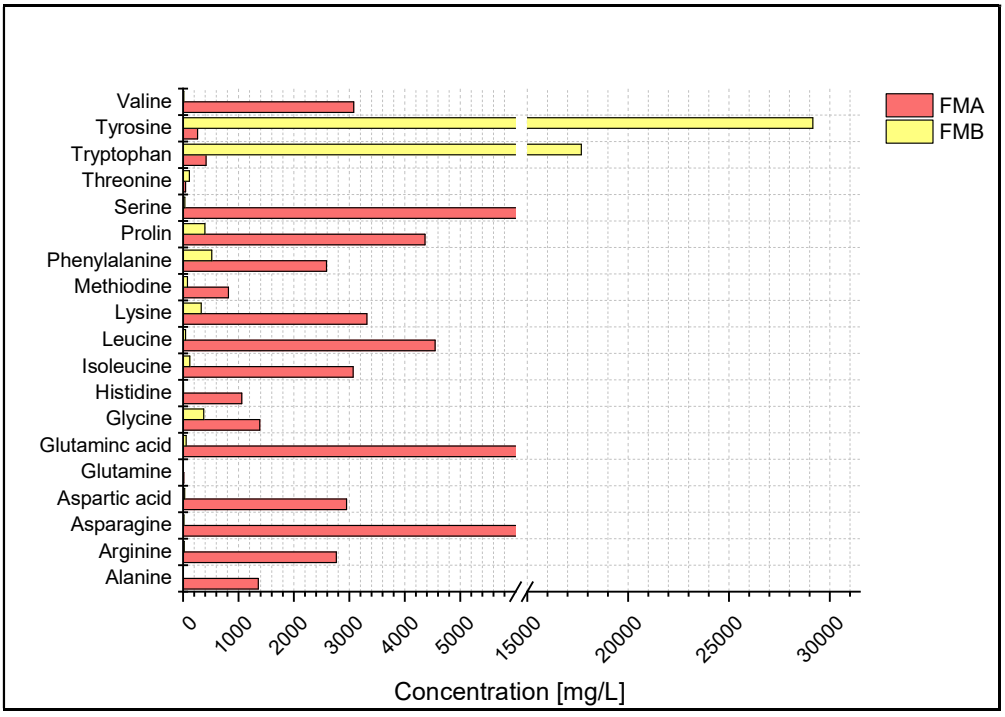


Figure 36 : Amino acid concentration in feed medium

Figure 37 depict the metabolic activity taking place in the CHO DG44 cells. The different metabolic pathways taking place in the cell are enlisted along with the precursors and the end product of the cell cycle in CHO cells.

Steps involved in glycolysis and TCA cycle were briefly represented in the figure. The bar diagram shown on the outer membrane represents the concentration of the particular component in medium of the cultivation carried out in the 2 L bioreactor (FBT2_018). Each of these amino acids present in the extracellular medium was measured using RP-HPLC and is discussed in detail in section 5.9.

Figure 37 helped in visualizing the depleted amino acid in the supernatant of the samples

taken during the entire course of cultivation. Despite the daily feed day 3 onwards, most of the amino acid was consumed during the stationary phase (day 7 onwards) with an exception of glutamine which was consumed by the end of day 3. This data can be used to optimize the medium and the concentration of feed medium added to the bioreactor to further optimize the cell growth and protein production in the cultivation.

The total amount of each amino acid added to the production bioreactor was measured and calculated individually for the different scales. This data was used to estimate the cell specific amino acid consumption and the results were compared to the cell growth and the cell specific protein production to determine the bottleneck for medium optimization. The amount of amino acids fed to the different scale of bioreactor per day during the fed batch by addition of both FMA and FMB is enlisted Table 27 in Appendix section 5.5.

A detailed information on individual amino acid is present in Appendix section 5.9.

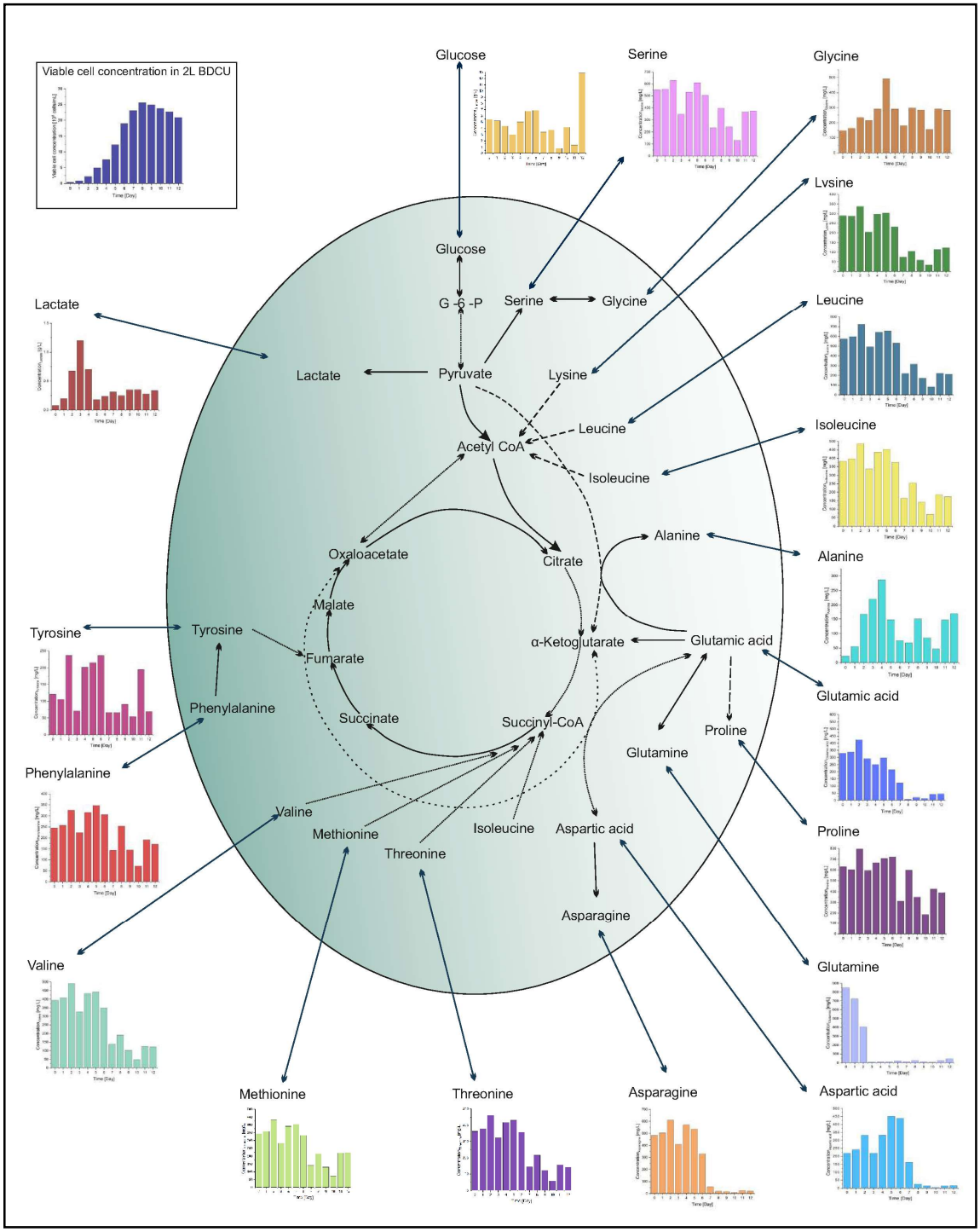


Figure 37 : Central carbon metabolism of CHO cells and the interaction with extracellular medium (Modified [85])

Serine as a main component of the product is not consumed during the cultivation, or can be converted from glycine or pyruvate. Alanine is converted from glutamate and not limited except the early beginning of the process. Valine and threonine are limited at day 7 usually as well as leucine and lysine, this four amino acids may be limit the product formation. This

can be prevented by a higher fed at day 6 or 7.

During the cultivation, the supernatant from the AMBR 15 cultivation run was stored and further analyzed in reverse phase HPLC to measure the 19 essential and non-essential amino acids required by the cells for growth and protein production. A heatmap was generated from these samples to study the concentration of each amino acid in the supernatant and the cells specific consumption of the amino acid during the cultivation was calculated using the equation:

$$q_{AA} = \left(\frac{(AA_1 + AA_{1+i}) - AA_2}{(t_2 - t_1)}\right) \cdot \frac{1}{\overline{x}} \qquad (16)$$

Where

q_{AA}:	cell specific amino acid consumption	[pg/cell/day]
AA_1:	Amino acid concentration at time interval 1	[mg]
AA_{1+i}:	Amino acid added during feeding at interval time 1	[mg]
AA_2:	Amino acid concentration at time interval 2	[mg]
$\overline{x}$:	Geometric mean of VCC	[10^6cells/mL]

Figure 38 represents the concentration of amino acid in the supernatant. The colour gradient ranged from red to green with a concentration range of 0 to 800 mg/L, with red being closer to 0 mg/L and 800 mg/L depicted as green in the heatmap. The depletion or accumulation of amino acid can be visualized in the figure below.

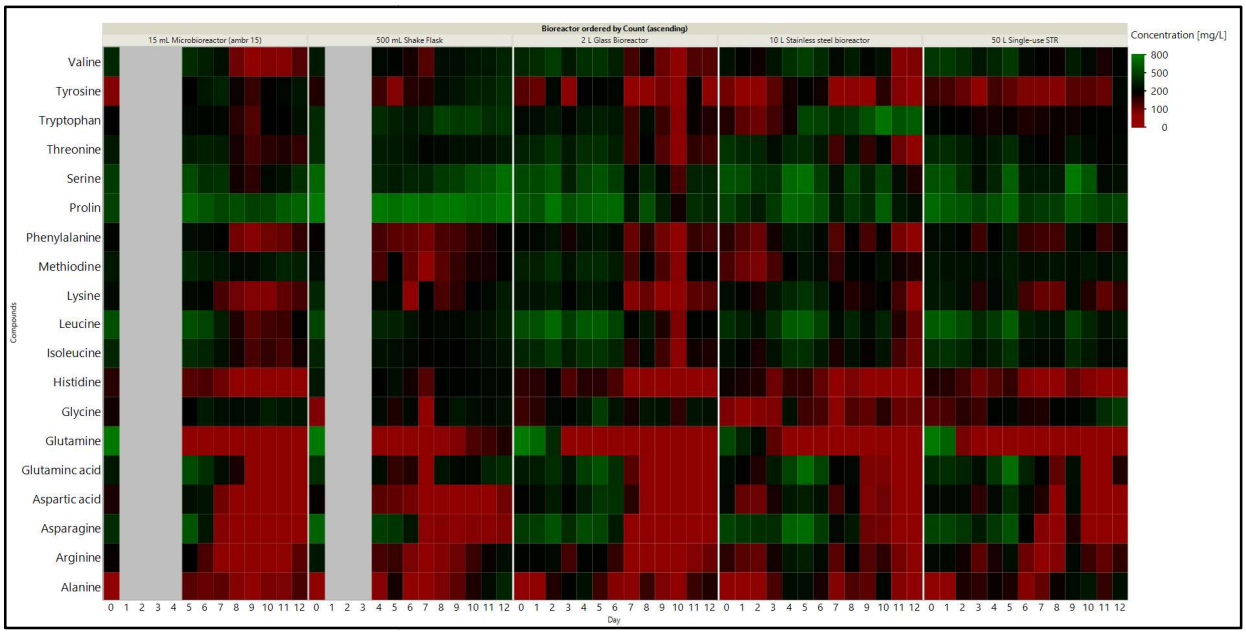


Figure 38 : Heatmap depicting the amino acid concentration in the supernatant

Figure 39 depicted the heat map showcasing the day to day cell specific consumption of each of the 19 amino acids in the different scales of bioreactor. The range of the heat map was from -25 pg/cell/day(red) to 50 pg/cell/day (green). In case the value is above or below the fixed ranges the intensity of the colour in the specific region intensifies.

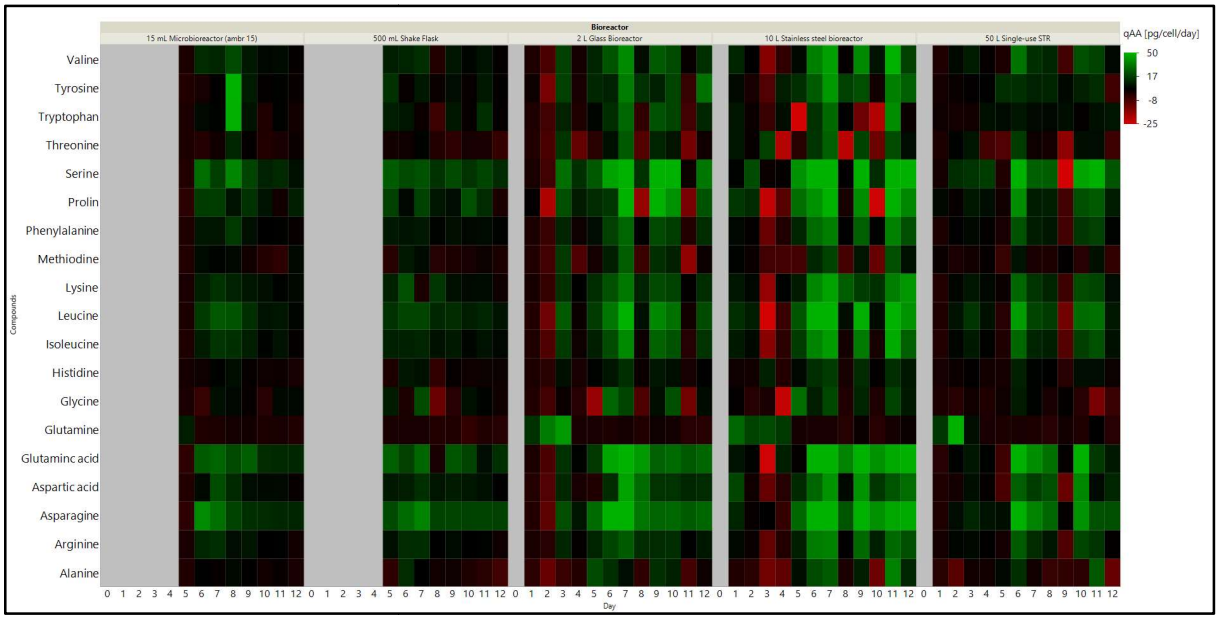


Figure 39: Heat map depicting day to day cell specific consumption

The discussion is divided based on the use of the amino acid for cell growth and protein production. However, it must be noted that the amino acids do not explicitly pertain to just these two roles. Each of these amino acids are discussed in detail in Appendix section 5.9.

For cell growth and proliferation: The amino acids predominantly used for cell growth and proliferation are tyrosine, phenylalanine and isoleucine. These amino acids are present in lower concentration in the genetic structure of the protein of interest.

It was also observed that isoleucine was consumed during the batch phase (until day 3) and accumulated till day 5 after which it was gradually metabolized till day 7 and the concentration in the medium remained constant even after the addition of Isoleucine to the culture; suggesting the use of isoleucine for cell growth and production of protein.

Similar to isoleucine, phenylalanine depletion results in cell growth arrest [86]. The initial concentration of phenylalanine in the production medium was measured between 150 to 250 mg/L and was gradually consumed until end of batch phase and was accumulated in the

medium till day 5. The concentration was maintained throughout the duration of the cultivation. The consumption of the phenylalanine in the different scales of bioreactor demonstrated the use of it during the transition on day 7 and an increase in the peak again at the end of the cultivation.

Tyrosine, phenylalanine and isoleucine were extensively consumed during the lag and initial exponential phase of the cultivation, suggesting a strong correlation between the consumption of these amino acids and cell proliferation. They were supplemented via the feed medium and the concentration within the culture medium remained constant, indicating the use of these amino acids also for sustainability of cell growth along with protein production.

For protein production: From Figure 34, the most abundantly present amino acid in the desired protein was serine with a total percentage of 12.78%. Serine is known to be an essential precursor for the synthesis of proteins, lipids and nucleic acid [87]. Therefore, it was essential to have sufficient quantity of serine in the culture medium until the end of the cultivation. During the cultivation, the cells metabolized serine during the batch phase followed by accumulation in the medium until day 5 and further metabolised by the cells thereafter. The maximum cell specific consumption was measured on day 7/8. This was the point in the cell phase when they shifted from exponential phase to stationary phase. In spite of being constantly consumed by the cells, the amount of serine in the medium was sufficiently present for the cells growth and protein production.

From Figure 38, the extensively present amino acid in all the five scales was measured to be proline. The percentage of proline in the selected monoclonal antibody was 6.6% and therefore was important to be adequately present in the medium. Since, the concentration was sufficiently present in the spent medium, it was not a possible bottleneck source of medium optimization for this strain and cultivation.

Along with being used for cell proliferation; aspartic acid, asparagine and histidine were also used for protein production and were completely metabolized by the cells by day 7. These were fed continuously, but these amino acids were constantly metabolized by the cells for daily functioning of the cells and protein production. The cell specific consumption of aspartic acid and asparagine was elevated during the stationary phase of the cell growth where as histidine was constantly being consumed by the cells from the start of the cultivation to the end of it, suggesting the use of it in daily functionality and sustainability of

the cells. To improve the cell growth and protein production, histidine along with aspartic acid and asparagine could be further supplemented to the bioreactors before they were completely depleted, i.e., before day 7 of the cultivation.

The other abundantly present amino acids in the monoclonal antibody were valine (9.01 %), leucine (7.81 %), threonine (7.67 %) and glycine (6.31 %).

Deprivation of valine in the surrounding of the cells leads to a decline in the synthesis of both RNA and protein. The quantity of valine in the medium was above 100 mg/L and the cells were not exposed to starvation of valine in the medium. From Figure 39, the cell specific consumption of valine was constant, thereby indicating the constant metabolism of valine during the cultivation.

The quantity of threonine present in the production medium ranged from 350 mg/L to 450 mg/L and the amount added through the feed medium was 1.56 mg/L per day. The cells constantly metabolized threonine, however it was also known that threonine and glycine metabolism were connected and the metabolism of both may result in increased pyruvate level and thereby increase in the flux through TCA cycle [13]. The consumption of glycine and threonine may not be as high as the other amino acids but the consumption is comparatively significant on the day of peak cell specific productivity (day 10 /11) ; indicating the use of these compounds for initiating protein formation and the protein production.

In the production medium, the concentration of alanine and glycine was comparatively lower; however, 5.8 % and 6.6% of the total amino acid sequence in the desired protein was composed of these amino acids, respectively. These were supplemented to the culture via the feed media and were constantly consumed during the stationary phase by the cells.

The amino acids in the least quantity in the protein were tryptophan (1.65 %) and methionine (0.75 %). The quantity of methionine in the production medium ranged from 150 mg/L to 350 mg/L and the amount added during fed batch was calculated to be 22.92 mg per litre of culture volume. In all the 5 scale of bioreactors, a peak in the consumption of methionine was observed during cells transition from exponential phase to stationary phase. An increase in consumption was also observed at the peak of cells specific productivity, indicating the use of certain amount of methionine for the protein production. The impact of tryptophan has to be further studied to conclude the influence on cell growth and protein production. During the cultivation, tryptophan was steadily metabolized; however, the results weren't conclusively in favour of growth rate or protein production.

Table 11 lists the comparison of final monoclonal antibody concentration, integral viable cell concentration, maximum growth rate, cell specific productivity and cell specific amino acid consumption for the 5 different scale of bioreactors. Based on historic data of cell growth, day 7 was used for the comparison of comparison of the specific amino acid consumption. In most reactors the cells transitioned from exponential to stationary phase on day 7.

Table 11 : Comparison of output variables in terms of amino acid consumption

		AMBR 15	500 mL shake flask	2 L BDCU	Cplus 10L	50 L STR (SUB)
Final IVCC [10^6 cells* day/ml]		199.72	159.28	177.46	166.22	198.79
Final mAb [g/L]		3.54	2.38	2.87	3.99	2.75
Maximum growth rate [Day^{-1}]		Day 5	Day 6	Day 2	Day 3	Day 2
		0.72	0.40	1.04	0.91	1.66
Maximum Cell specific productivity [pg/cell/day]		Day 12	Day 6	Day 10	Day 9	Day 11
		86.30	34.55	93.44	191.14	86.34
Average Cell specific productivity [pg/cell/day]		19.26	14.15	17.10	23.02	15.68
Cell specific amino acid consumption [pg/cell/day]	**Alanine**	2.73	4.14	6.83	25.34	1.36
	Arginine	13.78	13.54	25.90	36.56	13.45
	Asparagine	31.72	36.96	60.30	84.91	36.69
	Aspartic acid	21.74	14.30	45.81	41.52	17.36
	Glutamic acid	28.37	28.78	65.26	93.27	39.61
	Glycine	7.43	21.12	19.11	19.82	4.66
	Histidine	4.74	7.68	14.27	16.61	5.02
	Isoleucine	15.20	11.91	38.78	43.82	11.56
	Leucine	24.47	19.03	57.24	73.82	19.19
	Lysine	14.74	15.81	33.78	23.86	15.13
	Methionine	4.965	6.40	26.18	18.76	8.21
	Phenylalanine	8.35	9.42	28.57	35.36	22.79
	Proline	17.30	18.46	67.71	73.81	10.07
	Serine	17.65	23.13	67.06	88.91	27.13
	Threonine	1.45	4.75	24.90	26.85	1.81
	Tryptophan	4.77	2.18	28.93	29.68	6.25
	Tyrosine	3.91	9.12	34.76	42.71	11.35
	Valine	12.27	13.78	38.70	45.06	13.96

The final integral viable cell concentration of 15 mL microbioreactor and the 50L single-use bioreactor was maximum with a value of 199.72 x 10^6 cells*day/mL and 198.79 x 10^6 cells*day/mL. The 150 mL shake flask has the least amount of IVCC (159.28 x 10^6 cells*day/mL) due insufficient control of the process parameters such as pH and DO within the environment of the culture.

The final titre concentration in the 10 L stainless steel bioreactor reached a maximum value of 3.99 g/L.

The highest cell specific protein production was found in 10 L reactor, followed by the AMBR 15. The lowest was found in STR 50 with the highest growth rate. Comparing the 10 L and the 50 L runs one should indicate the consumption for growth and the other the amino acid consumption for product production.

The cell specific consumption of amino acid reached the peak value on day 7 during transition from exponential phase to stationary phase. This resulted in determining the estimate of maximum cell concentration during the cultivation and initiation of protein production. The maximum consumption of all the amino acids was observed in the 10L bioreactor and this corresponds to the increase in the protein concentration at the end of the cultivation. The consumption of the amino acids in the 2 L bioreactor and the 50 L single-use bioreactor were comparable except for glutamine, methionine, proline, threonine, tryptophan and tyrosine. These could be related to the difference in IVCC at the end of the cultivation. However, it must be noted that these values are only for the days it reached the peak value. Most of these amino acids are constantly consumed at lower concentration throughout the cultivation.

To summarize, the amino acid can be divided based on their metabolism for cell growth and protein production. The addition of feed medium can be further optimized by increasing the concentration of amino acids such as glutamine, aspartic acid, asparagine and histidine. These were the important amino acids that were consumed by day 7 and even after addition of these to the bioreactor, the cells constantly consumed them. Therefore, the culture medium can be supplemented with these compounds and the medium can be further optimized.

3.7 Alteration in feeding strategy

Pertaining to the above discussion, the amount of feed medium added to the bioreactor was varied to study the impact of increase of amino acid on the cell growth and protein production.

The first test of alternating feeding strategy was tested in shake flask wherein the set of shake flask triplicates was used to explore the influence of delayed feeding on cell growth and protein production. The feed media was added after 96 hours instead of the 72 hour

period.

Figure 40 represents the average of triplicate shake flasks offline data measured during the course of the cultivation. The maximum VCC of 22.16 x 10^6 cells/mL was reached on day 7. The cells remain in stationary phase until day 10 and eventually transient into the death phase. The VCC at the end of the cultivation was 19.33 x 10^6 cells/mL with a viability of 92%. The final titre was measured to be 2.46 g/L.

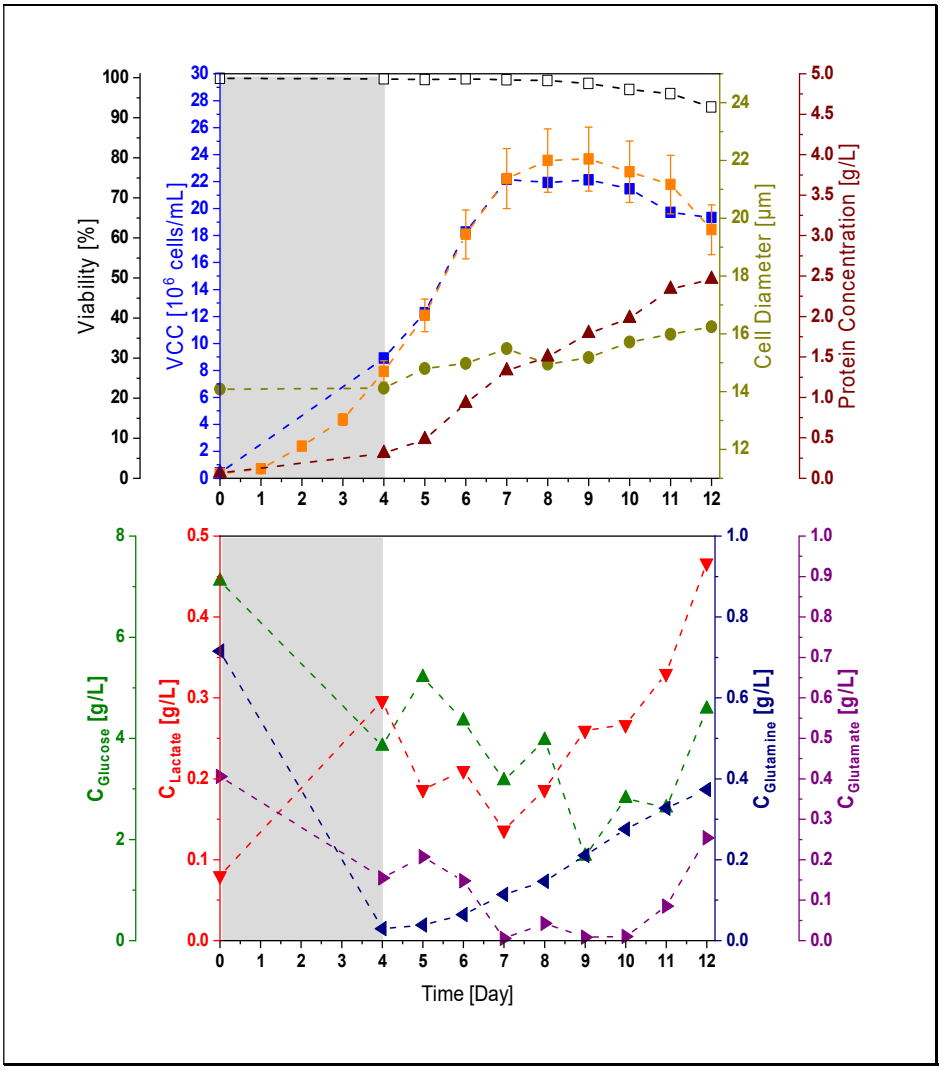


Figure 40: Offline analytics measured during cultivation in 500 mL shake flask with delayed feeding (FBT2_012). Shown is the viability (black), VCC (blue), VCC golden batch (orange), cell diameter (olive), protein concentration (maroon) and concentration of glucose (green), lactate (red), glutamine (navy blue), glutamate (purple)

Glucose was sufficiently present in the supernatant, indicating no substrate limitation occurred during the entire course of the cultivations. The lactate production and consumption followed a similar path to the cultivation FBT2_008, with consumption of

lactate occurring until day 7 followed by accumulation in the medium. The glutamine was completely consumed in the batch phase and was accumulated in the medium during the next 9 days. On the other hand, glutamate was supplemented to the bioreactor with the feed medium and it was consumed by day 7 and was negligibly present in the medium until day 11. On comparison to the protein produced during the cultivation, it was observed that the depletion of glutamate resulted in lower rate of production of the protein.

The growth of cells was comparable in both set of triplicates of 500 mL shake flask with start of feed on day 3 and day 4 (refer to Section 3.2.1) as they both contained the same amount of glucose and glutamine at the beginning of the cultivation. However, concentration of protein was negatively affected by the limitation of glutamate in the medium caused due to delayed feeding.

To study the impact further, AMBR 15 was used to conduct the study and the volume of FMA and FMB added to each reactor was calculated based on the working volume in the reactor on the particular day. The percentage of FMA and FMB added to the set of 15 mL microbioreactors is listed in Table 12, i.e., the working volume in control bioreactor on day 3 was 10 mL and the amount of FMA and FMB added was 3.98 % and 0.40 % of 10mL respectively.

Table 12 : Percentage of feed medium added to each scale of bioreactor

	Control feed		Double feed on day 5		Gradual increase of feed		Gradual decrease of feed	
	FMA (%)	FMB (%)	FMA (%)	FMB (%)	FMA (%)	FMB (%)	FMA (%)	FMB (%)
Day 3	3.98	0.40	3.98	0.40	3.98	0.40	10.85	1.09
Day 4	3.81	0.38	3.81	0.38	4.81	0.48	9.96	1.00
Day 5	3.66	0.37	7.32	0.74	5.66	0.57	9.08	0.91
Day 6	3.52	0.35	3.52	0.35	6.52	0.65	8.22	0.82
Day 7	3.36	0.34	3.36	0.34	7.36	0.74	7.36	0.74
Day 8	3.22	0.32	3.22	0.32	8.22	0.82	6.52	0.65
Day 9	3.08	0.31	3.08	0.31	9.08	0.91	5.66	0.57
Day 10	2.96	0.30	2.96	0.30	9.96	1.00	4.81	0.48
Day 11	2.85	0.29	2.85	0.29	10.85	1.09	3.98	0.40

Note: The bioreactors are represented by following alphabets for ease of understanding:
Control feed: A
Double feed on day 5: B
Gradual increase of feed: C
Gradual decrease of feed: D

The offline measurements taken during the course of cultivation were depicted in Figure 41.The green dotted line represents the control bioreactor; blue line depicts the results from

the bioreactor which was fed with double the amount of FMA and FMB on day 5; purple line represents the results from the bioreactor with gradual increase in the feed medium concentration and the red line depicts the results from the bioreactor initially fed with high concentration of FMA and FMB and thereafter a gradual decrease of feed medium in the culture.

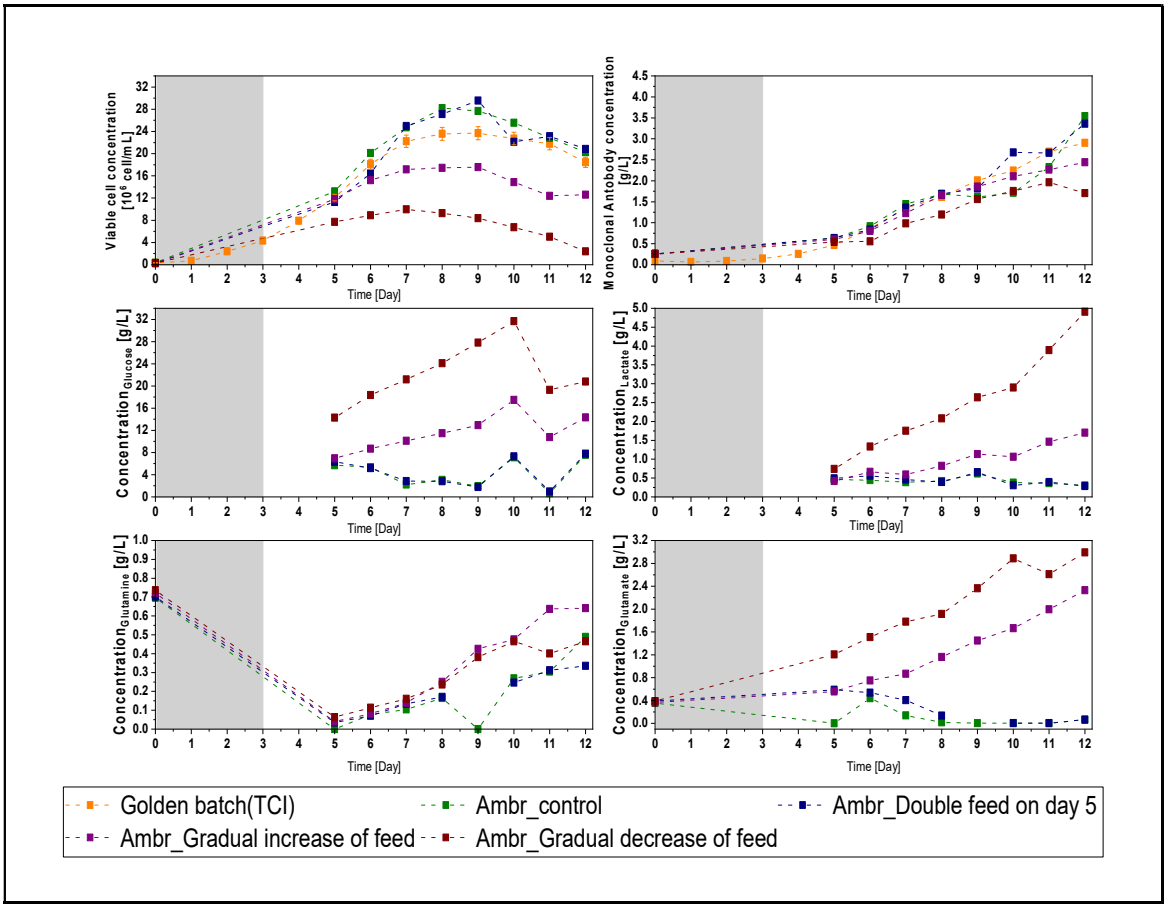


Figure 41 : Offline analytical measurement of bioreactors with different feeding strategy

The bioreactors with control feeding and double feeding on day 5 were comparable to the golden batch, indicating no significant influence of increasing the feed on day 5 occurred on the cell growth or the protein production. The metabolite consumption in the two sets of bioreactor followed the same pattern and there was no switch in the metabolism in terms of glucose, glutamine and glutamate.

A negative impact on the viable cell concentration was observed for bioreactors with gradual increase and gradual decrease of the feed (Figure 41). The cell concentration in the bioreactor with gradual increase in feed medium percentage was comparable until day 6, after which the growth rate of the cells reduced and the cell concentration reached the

stationary phase with the maximum cell concentration of 17.57 x 10^6 cell/mL and a final protein concentration of 2.44 g/L. The metabolite concentration increased at the end of cultivation. It was to be noted that glutamine was not added to the bioreactors during the fed batch phase but the amount of glutamine at the end of the process was measured to be 0.64 g/L, this amount was twice as high as the glutamine amount in the bioreactor with control feed. The glutamate amount in the bioreactors also increases to 2.33 g/L. It can be hypothesized that a reverse reaction took place in the CHO cells and a fraction of glutamate was used for production of glutamine in the bioreactors.

As the cultivation process parameters in the reactors was constant, it can be assumed that the concentration of the cell count would have been comparable in all the bioreactors with the same feed strategy. The difference in the different reactors occurred due to the variation of amount of feed medium fed to the different bioreactors.

In case of the bioreactor fed with high percentage of FMA and FMB, the cell growth was stunted and the maximum cell concentration reached by the bioreactor was 9.98 x 10^6 cell/mL and the final protein concentration was measured to be 1.7 g/L. The glucose amount in the bioreactor increased due to the addition of FMA and the value reaches a peak of 31.67 g/L. This increased the osmolality of the medium, in turn reduced the uptake of necessary nutrients by the cell. The increase in feed medium also resulted in stressing the cells and over production of lactate at the end of the cultivation. This also contributed in reduced cell count and protein production in the culture.

Apart from the measurement of the offline analytics, the supernatant was used to measure the amount of amino acid present in the medium and amount consumed by the cells was calculated based on equation 16. The results were represented in Figure 42 and Figure 43.

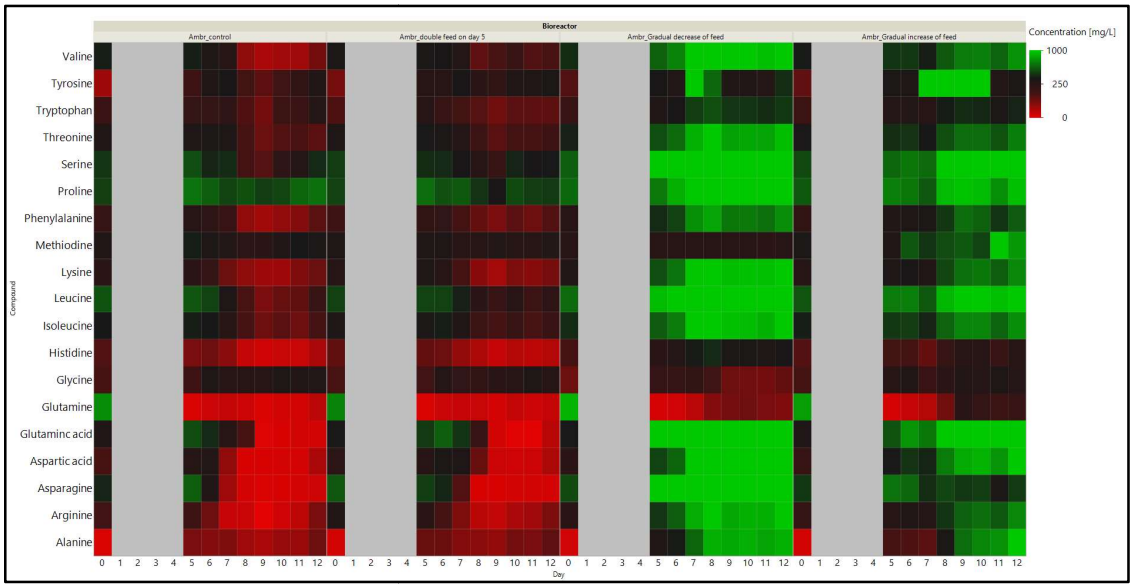


Figure 42 : Heat map depicting the amino acid concentration in the supernatant for different feeding strategy

Figure 42 represented a heat map generated from the measurement of the amino acid present in the supernatant. A colour gradient was used to depict the variation in the data. The heat map presented the concentration of each amino acid and it ranged from 0 mg/L to 1000 mg/L; with red (0 mg/L) representing the least concentration to green representing the most (1000 mg/L). If the values exceeded the range, the colour intensified further.

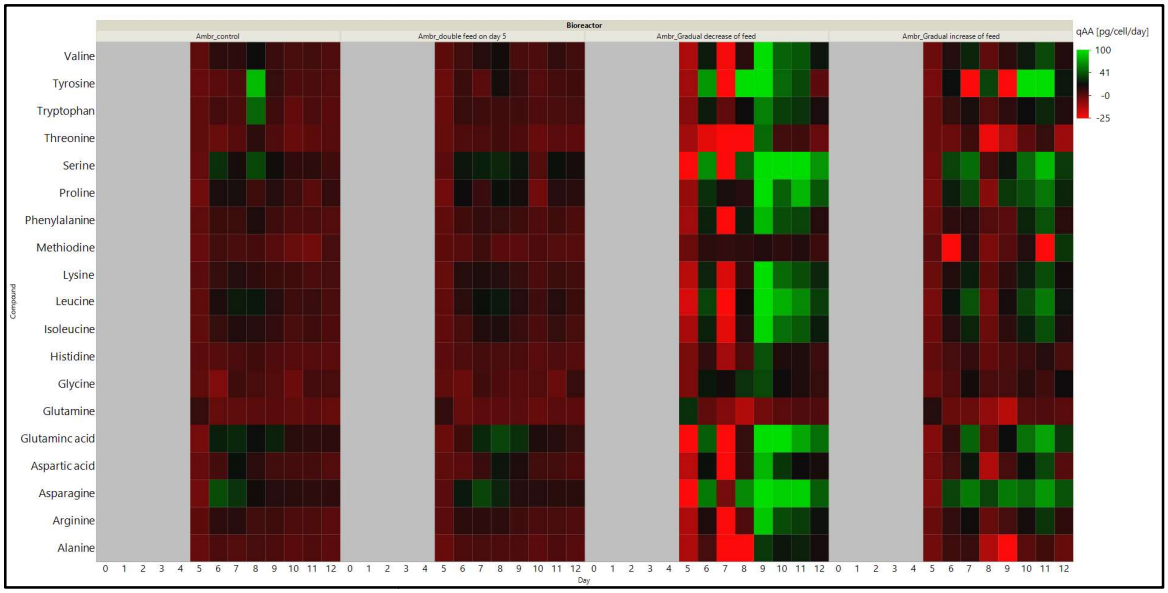


Figure 43 : Heat map depicting the cell specific amino acid consumption for different feeding strategy

Figure 43 was a heatmap generated for the visualization of the day to day cell specific

consumption of the amino acid in the bioreactors with different feeding strategy. The map ranged from -25 pg/cell/day (red) to 100 pg/cell/day (green).

As prefigure 42, the concentration of the amino acid in the bioreactor with control feed and double feed on day 5 was comparable and no significant change was visualized. The cell specific consumption of the amino acid (Figure 43) was also comparable in the two set of bioreactors, suggesting doubling the volume of feed only on one day was negligible and did not have an impact on the output data.

In the bioreactors with gradual increase and decrease of the feed medium percentage, the data indicated sufficient amount of all the amino acids present in the bioreactors. In both cases of Bioreactor C and D, alanine continued to be accumulated in the medium. The average cell specific consumption in Bioreactor "D" remained constant at the end of the cultivation with at an average of 25 pg/cell/day whereas Bioreactor "C" hardly consumes any alanine during the stage, indicating cells were unable to consume alanine for protein production resulting in low titre of 1.7 g/L.

From the previous segment, the quantity of a glutamine, aspartic acid, asparagine and histidine was negligible in the bioreactor with control feed and this was rectified in the Bioreactor C and D. Both these set of bioreactors had sufficient amount of aspartic acid, asparagine and histidine. Even with sufficient amount of amino acid in the supernatant the cell growth and the protein production was half the expected value. This was due to the presence of a few amino acids toxic to the cells in high concentration (such as leucine, methionine, phenylalanine, threonine, tryptophan and tyrosine [88]). This could be rectified by addition of only the specific amino acids to the bioreactor.

An interesting outcome from the study was the estimation of the cell specific amino acid consumption, visualized Figure 43, the metabolism of the amino acids was low until day 8 in bioreactors with gradual decrease of the feed medium percentage; suggesting the cells consumed the amino acids after the percentage of medium fed was 6.52 % the total volume for FMA and 0.65 % the total volume for FMB. In case of the bioreactors with gradual increase of feed medium, the negative effect of feed medium was observed only after the percentage of FMA increased above 6.52 % and FMB above 0.65 % the total volume of the bioreactor. This could be further used to optimize the percentage of feed medium added to the reactor in order to avoid accumulation of amino acids that can be toxic to the cells in higher quantity.

Table 13 : Comparison of output variables in terms of amino acid consumption in different feeding strategy

			Control Feeding (A)	Double feed on Day 5 (B)	Gradual increase of feed (C)	Gradual decrease of feed (D)
IVCC [10^6 cells* day/ml]		Value	199.72	188.37	137.05	73.33
Final mAb [g/L]		Value	3.54	3.36	2.44	1.70
Maximum Growth rate [Day^{-1}]		Day	5	5	5	5
		Value	0.72	0.73	0.75	0.66
Maximum Cell specific productivity [pg/cell/day]		Day	12	10	8	7
		Value	86.30	65.66	45.63	90.34
Average Cell specific productivity [pg/cell/day]		Value	19.26	18.43	17.69	15.64
Cell specific amino acid consumption [pg/cell/day]	**Alanine**	Day	8	8	7	9
		Value	5.95	4.08	9.62	33.43
	Arginine	Day	7	7	11	9
		Value	13.78	12.79	32.36	91.11
	Asparagine	Day	6	7	7	9
		Value	39.00	36.83	58.50	211.93
	Aspartic acid	Day	7	8	7	9
		Value	21.74	22.85	23.12	83.36
	Glutamic acid	Day	7	8	11	9
		Value	28.37	36.75	75.82	206.62
	Glutamine	Day	5	5	5	5
		Value	10.29	10.53	13.20	14.41
	Glycine	Day	7	7	7	9
		Value	7.43	7.27	18.86	38.19
	Histidine	Day	8	7	11	9
		Value	7.12	6.10	14.46	40.69
	Isoleucine	Day	7	8	11	9
		Value	15.20	15.80	39.70	97.12
	Leucine	Day	7	8	11	9
		Value	24.47	23.72	58.08	142.71
	Lysine	Day	7	7	11	9
		Value	14.74	15.81	39.26	107.73
	Methionine	Day	6	7	12	9
		Value	6.43	8.12	32.79	15.09
	Phenylalanine	Day	8	7	11	9
		Value	16.10	10.68	40.73	84.13
	Proline	Day	7	8	11	9
		Value	17.30	21.72	56.03	142.35
	Serine	Day	8	8	11	9
		Value	37.65	27.86	84.09	201.98
	Threonine	Day	8	8	11	9
		Value	12.20	7.06	10.28	51.08
	Tryptophan	Day	8	7	11	9
		Value	9.52	8.10	27.82	59.74
	Tyrosine	Day	8	8	11	8
		Value	20.37	18.00	139.02	144.99
	Valine	Day	8	8	11	9
		Value	20.37	19.16	38.12	103.59

The integral viable cell concentration at the end of the cultivation varied drastically for the Bioreactor C and D when compared to Bioreactors A and B. The IVCC for Bioreactor D was 73.33 10^6 cells* day/mL with a final protein concentration of 1.70 g/L; even with low cell count and protein concentration, the cell specific productivity was measured to be 15.64 pg/cell/day, indicating the comparatively lower number of cells actively produced the protein of interest.

The amino acid consumption in Bioreactor A and B reached a peak value between days 6 to 8 whereas the metabolism of amino acid varied in Bioreactor C and D. In Bioreactor C, the metabolism of alanine, asparagine, aspartic acid and glycine reached the peak consumption on day 7 and the remaining amino acids reach a peak metabolism by cells on day 11. In case of Bioreactor D, most of the amino acids reached a peak metabolism on day 9 with values five to eight times higher than the consumption rate of control bioreactor. The increase in the uptake of amino acids in bioreactor D suggested feed medium composition can be further improved to increase the titre at the end of the cultivation without disruption the cell growth of the culture.

Also, from the heatmap it was concluded that FMA concentration of above 6.52 % the total working volume and FMB concentration of 0.62 % of total working volume was detrimental to the cell growth and protein production. Therefore, based on the information, feed medium can be optimized such that optimal percentage of amino acid is added to the cultivations and is restricted to the norms mentioned above.

4 Summary and Outlook

The primary aim was to test the scalability and transferability of processes in different scales and types of bioreactors. Based on the scalability, the process parameters (pH and DO) and medium composition were optimized to increase the final product concentration.

The different bioreactors, multi-use and single-use bioreactors were included in the study, namely: 500 mL shake flask, 15 mL and 250 mL automated micro-bioreactor (AMBR 15 and AMBR 250), 2 L glass bioreactor, 10 L stainless steel bioreactors and 50 L single-use stirred tank reactor.

The different bioreactors (excluding the shake flask) were geometrically similar and had comparable mixing characteristic. Thus, fulfilling the fundamental prerequisite for process transfer between different cultivation platforms.

For successful transfer of the process, the maximum viable cell concentration (VCC) would be in the range of 17- 26 x 10^6 cells/mL with final product concentration ranging from 2.5 g/L to 5 g/L. The transfer of the process was carried out successfully across different scales of bioreactors with an exception of 250 mL microbioreactor (AMBR 250) due to technical malfunction of the disposable bioreactor used for the cultivation. Despite the deviation from the golden batch, the VCC in AMBR 250 reached a maximum value of only 20 x 10^6 cells/mL. Thus, concluding the successful transfer of the process in terms of the viable cell concentration in the 6 scales of bioreactors used.

The other factor considered for the successful transfer of the process was the final monoclonal antibody concentration. This concentration ranged from 2.4 to 4 g/L in different bioreactors, with maximum titre of 4 g/L in the 10 L reactor and the lowest of 2.4 g/L in the AMBR 250. However, on comparison with the integral viable cell concentration, a direct correlation between cell concentration and final monoclonal antibody production was not established. The production of protein in the culture is not directly related to the IVCC but is based on the process condition, such as DO and medium composition.

Several factors contributed to positively or negatively impact the cell growth and protein production such as delayed feeding, alteration in DO and pH, influence of carbon dioxide, alteration of production and feed medium.

Delaying the feed by a day in shake flask resulted in negative influence on the titre due to limitation of glutamate in the medium, which is essential for product production. However,

the impact on the cell growth in the shake flask was negligible. The initial assessment of the relation between glutamate and titre production resulted in studying the impact in greater detail across all scales of bioreactors. It was observed that the protein production was directly correlated to the consumption of glutamate, thereby providing a scope of improvement in the process to increase the final titre.

The other factor considered to have a direct influence on cell growth was the combination of pH and concentration of carbon dioxide (a gas used to maintain the pH in the bioreactors). The influence was studied in 2 L glass bioreactors, where two cultivations were carried out with the main difference being the supply of CO_2 gas. The maximum flow rate of carbon dioxide used to maintain the pHin the altered gas supply source was 10 ccm and the cell growth was comparable to the golden batch; where as in FBT2_014, the cell growth was significantly lower and the system required 2.5 times higher amount of CO_2 (25 ccm).

During cultivation in 10 L stainless steel bioreactor, it was observed that the cell growth was negligible during the batch phase and exponentially grew during the fed batch. It was hypothesized that the longer lag phase was due to the combination of high DO percentage at the beginning of cultivation and change in volumetric mass transfer coefficient caused by dissimilar geometric ratio of the bioreactor when compared to the other reactors. The influence of high DO on the cell growth was further studied in AMBR 15 reactor. However, due to geometric dissimilarity between the two bioreactors, a direct correlation between the high DO, k_La and cell growth could not be established. This would have to be further studied by understanding the change in the volumetric mass transfer coefficient in the 10 L bioreactor by altering the agitation speed, aeration and volume within the bioreactor.

The findings from the 2 L and 10 L bioreactor opened the possibility of optimizing the process in terms of the dissolved oxygen concentration (set point 40 %, 60 % and 80 %) and pH (set point of 6.9, 7.1 and 7.3).

The bioreactors with DO set point of 40 % and 80 % contained comparable integral viable cell concentration at the end of the cultivation, however, the average cell specific productivity in the bioreactor with DO set point of 40 % was higher, suggesting more cells in bioreactors with set point 40 % were actively producing the desired protein. The final titre in all three bioreactors ranged between 3.05 g/L to 3.54 g/L and it was concluded that the reduction in the DO set point does not have a negative effect on the cells or the product quantity. The DO set point for the production scale can be reduced to 40 % to produce the

same amount of protein with reduced damage to the cells due to oxygen driven free radicals.

The bioreactors with pH set point of 7.1 and 7.3 had comparable cell growth and final titre concentration. However, the average cell specific productivity was comparatively higher in pH 6.9 than pH 7.3. This implies that the amount of cells producing the protein were comparatively more active in pH 6.9. It was deduced that alkaline and acidic parameters not only influence the overall metabolism but also cell growth and productivity. The set point of 7.1 was ideal for the cultivation and it can be increased up to 7.3 with positive influence on cell proliferation and no negative effect on the product concentration.

For the future cultivations in this process, the set points for scale up can be set at pH of 7.1 and DO of 40 %.

The next step for optimizing the process was to study the impact of carbon (glucose) and nitrogen (glutamine and glutamate) source on the cell growth and protein production by altering these concentrations in the main cultivation medium. The result depicted that the production medium can be enhanced by altering the initial concentration of glutamine and glucose to range between 0.6 to 1.2 g/L and 6 to 12 g/L, respectively. Glutamine can also be fed to the bioreactor individually as a bolus feed to meet the demand of cultivated cells during stationary and death phase.

During the entire course of the cultivation, the amino acid concentrations in the supernatant were measured using RP-HPLC to further understand the critical compound to improve the cell growth and product quantity. The amino acids were divided based on their metabolism for cell growth and protein production. However, it was also noted that the roles were interchangeable for few compounds.

The amino acids predominantly used for cell growth are glutamine, tyrosine, phenylalanine and isoleucine, whereas, the remaining 15 amino acids are used for protein production and regulation of basic functionality within the cells. Amino acids such as glutamine, aspartic acid, asparagine and histidine were completely metabolised by day 7 and a bolus of these could potentially be supplemented to the culture medium to further improve the cell growth and protein production.

The study paved way to test the impact of varying the percentage of feed medium A (FMA) and feed medium B (FMB) during cultivation. From the results it was concluded that FMA concentration of above 6.52 % of the total working volume and FMB concentration of 0.62 %

of the total working volume was detrimental to the cell growth and protein production. Therefore, based on the information, feed medium can be further optimized such that optimal percentage of amino acids to be added to the cell culture increase to 6.52% of the total working volume for FMA and 0.62% of the total working volume for FMB.

This project highlights the success of the transfer and scalability of cultivation process in both single-use and multi-use bioreactor ranging from a working volume of 15 mL to 50 L. Each scale of bioreactor further increased the understanding of the impact of each parameter on the end product. To further improve the cultivation process, a Design of Experiment (DoE) can be structured to test the impact of pH and DO on cell growth as a combined entity. Along with the process parameter, the production medium composition and the feed media percentage can also be varied to improve growth and product production. The knowledge from fed-batch process can be further used to develop a continuous cultivation to extract maximum product from the cultures. Along with this, a study to optimize the preculture can further help improve output of the cultivation.

5 Appendix

5.1 List of experiments

Table 14 : List of experiments represented in the project report

Cultivation Number	Bioreactor scale	Purpose
FBT2_008	Shake flask (500 mL)	Scaling of process
FBT2_012	Shake flask (500 mL)	Testing the influence of delayed feeding
FBT2_014	BDCU (2L)	Scaling of process
FBT2_018	BDCU (2L)	Scaling of process and testing the influence of changed gas supply on the cell growth
FBT2_015	Cplus (10L)	Scaling up of process
FBT2_020	STR 50 (50 L)	Scaling up of process
FBT2_023	AMBR 250 (250 mL)	Scaling down of process
FBT2_016	AMBR 15 (15 mL)	Testing the influence of pH on the process
FBT2_021	AMBR 15 (15 mL)	Testing the following: • Influence of dissolved oxygen concentration • Influence of altered production medium • Impact of changed feeding strategy

5.2 List of chemicals and consumables

Table 15: List of consumables

Description	Product	Vendors
Bottle Top Vacuum Sterile filter	1L Filter System 0,1 µm	Corning, USA
CEDEX sample cup	Cedex HiRes sample cup	Roche Innovatis AG, Switzerland
Disposable cutting blade for Sterile tube welder	BioWelder® Disposable Blades	Sartorius Stedim Biotech GmbH, Germany
	BioWelder® TC Disposable Blades	Sartorius Stedim Biotech GmbH, Germany
Erlenmeyer cell culture flasks with baffle	500 mL Corning® Erlenmeyer cell culture shake flask	Corning, USA
Falcon Tubes	15 mL Centrifuge Tubes	Corning, USA
	50 mL Centrifuge Tubes	Corning, USA
Glass Bottles	500 mL DURAN® Laboratory Glass bottles	DWK Life Sciences, Germany
	1000 mL DURAN® Laboratory Glass bottles	DWK Life Sciences, Germany
	5000 mL DURAN® Laboratory Glass bottles	DWK Life Sciences, Germany
Hose connector	Rotilabo® Hose connectors	Carl Roth GmbH Germany
HPLC Vials	0.3 mL PP transparent conical insert	VWR International, USA
Serological pipettes	Serological pipette 2 mL	Corning, USA
	Serological pipette 5 mL	Corning, USA
	Serological pipette 10 mL	Corning, USA
	Serological pipette 25 mL	Corning, USA
	Serological pipette 50 mL	Corning, USA

Single use tubes	Silicon tube (of varied sizes)	VWR International, USA
	C-Flex® 374	Saint-Gobain, France
	PharMed® BPT	Saint-Gobain, France
Sterile filter	Minisart® PES 0,22 µm	Sartorius Stedim Biotech GmbH, Germany
	Midisart® 2000 0,2 µm PTFE	Sartorius Stedim Biotech GmbH, Germany
	Sartofluor® Junior 0,2 µm	Sartorius Stedim Biotech GmbH, Germany
	Sartofluor® Mini Cartridge 0,2 µm	Sartorius Stedim Biotech GmbH, Germany
Syringe Filter	Millex® GV 4mm 0,22 µm	Merck KGaA, Germany
Syringes	BD Luer-Lok tip 20 mL syringe	BD Biosciences, USA
	Injekt® -F 1 mL	B. Braun Melsungen AG, Germany

Table 16: Equipment list

Description	**Product**	**Vendors**
Autoclave	VX-150	Systec GmbH, Germany
Autosampler	ASX-260 AutoSampler	Teledyne CETAC, USA
Biological safety cabinets	Safe 2020	Thermo Fisher Scientific, USA
	MSC-Advantage™	Thermo Fisher Scientific,USA
Bioreactors	Biostat ®BDCU	Sartorius Stedim Biotech GmbH, Germany
	AMBR ®250	Sartorius Stedim Biotech GmbH, Germany
	Biostat ®CPLUS	Sartorius Stedim Biotech GmbH, Germany
	AMBR ®15	Sartorius Stedim Biotech GmbH, Germany
Cell counter	CEDEX HiRes	Roche Innovatis AG, Switzerland

Centrifuge	Micro Star 17	VWR International, USA
HPLC Autosampler	Triathlon	Spark, Holland
HPLC Column (Protein A)	POROSTM A 20 µ m	Thermo Fisher Scientific, USA
HPLC Column (Reverse Phase HPLC)	Zorbax Eclipse Plus C18, 3,5µm	Agilent Technologies, USA
HPLC Column oven	Techlab T-1	Techlab GmbH, Germany
HPLC Degasser	3-Kanal-Degasser	SYKAM Chromatographie Vertriebs GmbH, Germany
HPLC Detector	Fluorescence Detector RF-10AxL	Shimadzu, Japan
HPLC Guard cartridge	SecurityGuardTM, GFC4000, 4x 3 mm	Phenomex Inc., USA
HPLC Guard cartridge holder	SecurityGuardTM Guard cartridge holder	Phenomex Inc., USA
HPLC Program	Open Lab Control Panel	Agilent Technologies, USA
	Clarity 5.0	DataApex Ltd., Czech Republic
HPLC Pump	Agilent 1200	Agilent Technologies, USA
HPLC System	Chromaster	VWR International GmbH
Incubator	Heracell™ 240	Thermo Fisher Scientific, USA
pH Meter	HI 211	Hanna Instruments, Kehl am Rhein
pH probe	OxyFerm FDA VP 225	Hamilton Robotics, USA
Pipette	accu-jet® pro	Brand, Germany
pO2 probe	EasyFerm Plus PHI VP 225 Pt100	Hamilton Robotics, USA
Sterile tube welder	Biowelder®	Sartorius Stedim Biotech GmbH, Germany
	Biowelder Total Containment	Sartorius Stedim Biotech GmbH, Germany
Vacuum pump	LABOPORT® Mini	KNF Neuberger GmbH, Germany
Vortex	Genie 2	Bender & Hobein GmbH, Germany

Water bath	Waterbath WNB Basic	Memmert GmbH + Co. KG, Germany
	AC 210 S	Sartorius Stedim Biotech GmbH, Germany
Weighing balance	Midrics 2	Sartorius Stedim Biotech GmbH, Germany
	CP12001S Top-loading Balance	Sartorius Stedim Biotech GmbH, Germany
YSI	YSI 2900 Select	YSI Inc.,Yellow Springs, Ohio, USA

5.3 Media preparation

Note: All the medium prepared for the cultivation are sterile filtered using 0.1µm pore size, bottle-top vacuum filters.

Table 17: Recipe for 1L of Seed medium

Component	Concentration	Supplier	Comment
ddH_2O	970 mL		
Cellca SMD/ CHOKO Stock culture medium	20.04 g/L	SAFC	Mix for 60 minutes
Sodium hydroxide pellet	0.24 g/L	Merck	Mix for 30 minutes
Sodium Bicarbonate	1.80 g/L	Sigma Aldrich	Mix for 15 minutes
ddH_2O	Make volume up to 1000 mL		
L-Glutamine [200 mM]	6 mM	Lonza, Switzerland	
MTX [500 µM]	15 nM	Sigma Alrich	Only up to passagen-3
pH adjusted to 6.9- 7.3 with 2 M NaOH			

Table 18: Recipe for 500 µM MTX stock solution

Component	Concentration	Supplier	Comment
1 M Sodium Hydroxide solution	100 mL	Roth	
Methotrexate hydrate (MTX)	22.7 mg	Sigma Aldrich	Add MTX to the 1 M NaOH solution

Filter the dissolved solution with sterile 0.1 µm filter into two sterile falcon tubes and aliquot them into 1 mL vials. Freeze the cryo vials at -20 °C

Table 19: Recipe for 1L of Production medium

Component	Concentration	Supplier	Comment
ddH_2O	970 mL		
Cellca PM/ CHOKO Production Medium	22.31 g/L	SAFC	Mix for 60 minutes
5 M Sodium hydroxide	6.2 mL/L	Merck	Added stepwise, Mix for 30 minutes
Sodium Bicarbonate	1.80 g/L	Sigma Aldrich	Mix for 15 minutes
ddH_2O	Make volume up to 1000 mL		
L-Glutamine [200 mM]	6 mM	Lonza, Switzerland	
pH adjusted to 6.9- 7.35 with 2 M NaOH			

Table 20: Recipe for 1L of FMA solution

Component	Concentration	Supplier	Comment
ddH_2O	850 mL		
Cellca FMA/ CHOKO FMA	154.12 g/L	SAFC	Mix for 60 minutes
Sodium hydroxide pellet	3.148 g/L	Merck	Mix for 30 minutes
ddH_2O	Make volume up to 1000 mL		
pH adjusted to 6.5- 6.8			

Table 21: Recipe for 1L of FMB solution

Component	Concentration	Supplier	Comment
ddH_2O	850 mL		
Cellca FMB/ CHOKO FMB	94.60 g/L	SAFC	Mix for 60 minutes
Sodium hydroxide pellet	32.10 g/L	Merck	Mix for 30 minutes
ddH_2O	Make volume up to 1000 mL		

pH adjusted to 11.0 – 11.4			

Table 22: Recipe for 1L of 400g/L of glucose stock solution

Component	Concentration	Supplier	Comment
ddH_2O	700 mL		
D-Glucose	400 g/L	Merck	Mix for 60 minutes
ddH_2O	Make volume up to 1000 mL		

Table 23: Recipe for 1L of 2% Antifoam solution

Component	Concentration	Supplier	Comment
ddH_2O	933.3 mL		
Antifoam C Emulsion (30%)	66.7 mL	Sigma	Mix for 60 minutes
Autoclave the solution at 121 °C for 20 minutes			

5.4 Seed culture

The steps involved in seed culture preparation are listed below:

1. 150 mL of Stock culture medium with 15 nM of MTX was pre-warmed at 36.8 °C for a minimum of two hours in 500 mL shake flask before the thaw of the vial and in another 15 mL centrifuge tube, 10 mL of stock culture medium with 15nM MTX was incubated at 2 – 8 °C for a minimum of two hours.
2. The vial was thawed to room temperature and transferred to the falcon tube containing 10 mL of medium under sterile condition.
3. The cell suspension was centrifuged at 190 xg for 3 minutes and the supernatant was decanted under the biological safety cabinet.
4. The cell pellet was gently re-suspended using the pre-warmed medium from the shake flask and the cell suspension was transferred to the shake flask.
5. 1mL of the sample was removed and the initial viable cell density was measured using CEDEX HiRes cell counter.
6. The flask was placed in an incubator with temperature set at 36.8 °C, CO_2 of 7.5% and shaker speed of 120 rpm (19mm,orbital diameter).
7. After three days the cells were split such that the initial viable cell density was 0.2 x 10^6 cells/mL using the formula:

$$Inoculum\ volume = \frac{150\ (mL) x\ 0.2\ (10^6 x\ cells/mL)}{MeasuredVCD(10^6 x\ cells/mL)}$$

Table 24: Process flow chart as per protocol [89]

Vessel name	**Cryo vial ↓ Shake flask 500mL**	**Shake flask 500mL**	**Shake flask 500mL**	**Shake flask 500mL**	**Shake flask 500mL**	**Production bioreactor**
Step	Cell Thaw n-5	n-4	n-3	n-2	n-1	Main culture
Passage	1	2	3	4	5	6
Medium	SMD with MTX	SMD with MTX	SMD with MTX	SMD	SMD	PM
Initial viable cell density [10^6 cells/mL]	0.2	0.2	0.2	0.2	0.2	0.3
Duration [Days]	3	3 - 4	3 - 4	3 - 4	3	12

5.5 Main cultivation

The process parameters for a standard cultivation is enlisted in the table below:

Table 25: Process parameters in production bioreactor

Process parameters	**Set point**
Initial working volume	66 % of total working volume
Aeration	0.03vvm
DO	60 %
Temperature	36.8 °C
pH	7.1 (control by aerating CO_2)

Glucose, feed medium and antifoam was added to the bioreactor until the end of the cultivation depending on the demand.

Addition of feed (FMA and FMB) started after 72 hours of cultivation time and the bioreactors were fed every 24 hours thereafter until day 11. Feed medium amount added was calculated using the following formulae.

Amount of feed medium A added = 42 (g/L) x Starting working volume (L)/ day

Amount of feed medium B added = 4.2 (g/L) x Starting working volume (L)/ day

The amount of FMA and FMB added to the different reactor on each day (day 3 onwards has been listed out in Table 26

Table 26: Amount of FMA and FMB added to different scales of bioreactor

Days	AMBR		Shake flask		2L BDCU		10L Cplus		50 L STR	
	FMA (mL)	FMB (mL)	FMA (mL)	FMB (mL)	FMA (mL)	FMB (mL)	FMA (mL)	FMB (mL)	FMA (mL)	FMB (mL)
3	0.40	0.04	4.0	0.4	55.44	5.6	277.2	28	1386	139
4	0.42	0.04	4.0	0.4	55.44	5.6	277.2	28	1386	139
5	0.39	0.04	4.0	0.4	55.44	5.6	277.2	28	1386	139
6	0.37	0.04	4.0	0.4	55.44	5.6	277.2	28	1386	139
7	0.36	0.04	4.0	0.4	55.44	5.6	277.2	28	1386	139
8	0.34	0.04	4.0	0.4	55.44	5.6	277.2	28	1386	139
9	0.33	0.03	4.0	0.4	55.44	5.6	277.2	28	1386	139
10	0.31	0.03	4.0	0.4	55.44	5.6	277.2	28	1386	139
11	0.30	0.03	4.0	0.4	55.44	5.6	277.2	28	1386	139

Table 27 : Amount of amino acids fed to the different scale of bioreactor per day

Amino acid	Amount in mg				
	AMBR 15	500 mL shake flask	2 L BDCU	10 L Cplus	50 L SUB (STR)
Alanine	0.546	5.430	75.255	376.273	1881.364
Arginine	1.112	11.063	153.339	766.697	3833.464
Asparagine	2.461	24.484	339.356	1696.779	8483.878
Aspartic acid	1.186	11.805	163.619	818.093	4090.435
Glutamic acid	2.544	25.316	350.885	1754.425	8772.069
Glutamine	0.006	0.057	0.792	3.959	19.789
Glycine	0.572	5.688	78.863	394.315	1971.204
Histidine	0.426	4.235	58.701	293.507	1467.528
Isoleucine	1.239	12.327	170.858	854.288	4271.317
Leucine	1.830	18.210	252.386	1261.931	6309.612
Lysine	1.347	13.400	185.736	928.678	4643.061
Methionine	0.333	3.307	45.843	229.214	1145.994
Phenylalanine	1.062	10.555	146.323	731.617	3657.565
Proline	1.772	17.621	244.252	1221.258	6105.894
Serine	3.145	31.291	433.698	2168.491	10842.427
Threonine	0.023	0.224	3.114	15.572	77.744
Tryptophan	0.909	8.730	121.982	609.912	3031.880
Tyrosine	1.330	12.713	177.832	889.162	4416.656
Valine	1.239	12.325	170.829	854.143	4270.702

Addition of glucose started after 125 hours and was fed to the bioreactor when the glucose concentration was below 5.0 g/L.

5 mL of sample was extracted every day for measurement of offline pH, viable cell concentration and viability using CEDEX HiRes cell counter. The remaining cell suspension was centrifuged at 190 xg for 5 minutes at room temperature and the supernatant was used to measure glucose, lactate, glutamine and glutamate using YSI 2900. The remaining sample was frozen and later used to measure protein (using Protein A chromatography) and amino acid concentration (using RP-HPLC).

5.6 Gassing in 2 L glass bioreactor

5.6.1 Aeration controlling DO in FBT2_014

The DO set point of 60 % was maintained during the cultivation and this was controlled initially by aerating a mixture of air and nitrogen. At the end of the batch phase, the cells required a mixture of pure oxygen and air to maintain 60 % DO. A small fluctuation in the DO concentration was observed due to addition of antifoam. This influence of antifoam on the DO was insignificant and the system was able to maintain the desired value throughout the cultivation. However, during the end of the cultivation, that is, day 9 onwards the cells began to die and did not require as much oxygen to maintain the DO. The aeration was switched back to a mixture of air and nitrogen to maintain the set point.

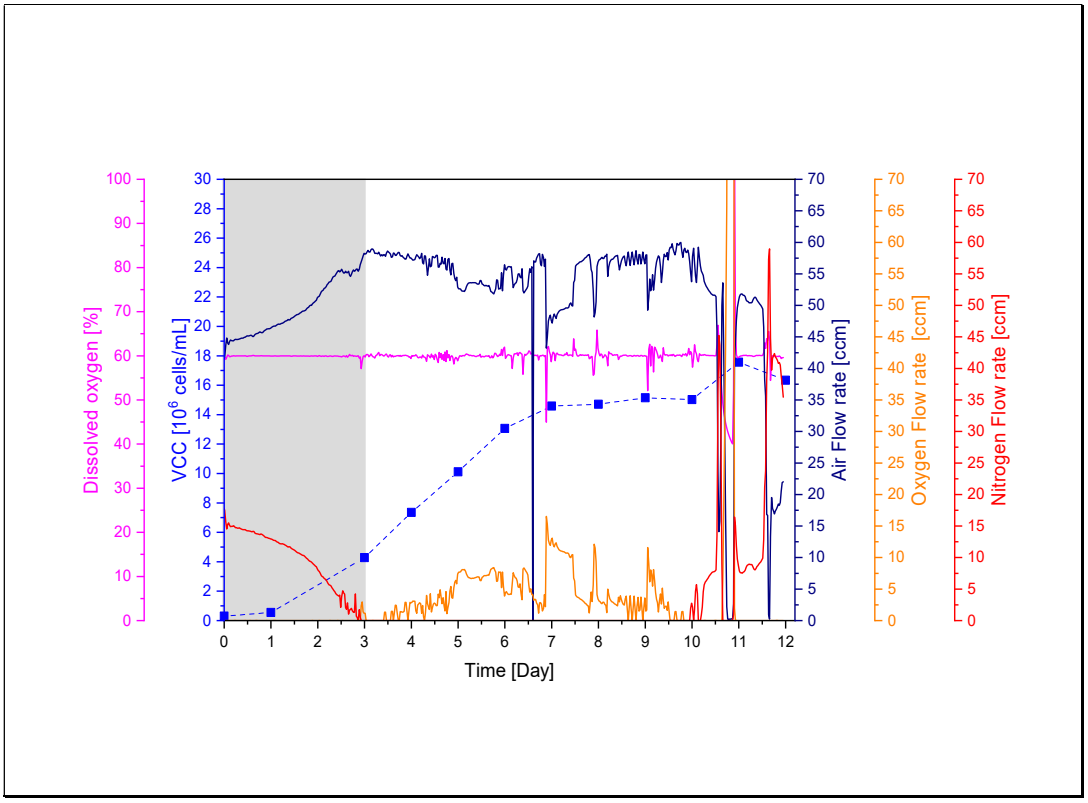


Figure 44: Aeration controlling DO within the 2L glass bioreactor (FBT2_014)

5.6.2 Aeration controlling DO in FBT2_018

Figure 45 showcases the gases involved in the maintenance of the DO. The gas mix of nitrogen and air was sparged into the system until day 3 and a mixture of air and oxygen was used for the maintenance of the oxygen concentration in the bioreactor to 60 %. The

deviation or fluctuation in the DO was due to addition of antifoam. On day 7, the amount of oxygen being consumed by the cells was more than what was supplied and therefore there was a switch between the mixing percentage of air and oxygen in the bioreactors. This is a positive sign as the cells are consuming more oxygen and therefore require more to sustain their growth.

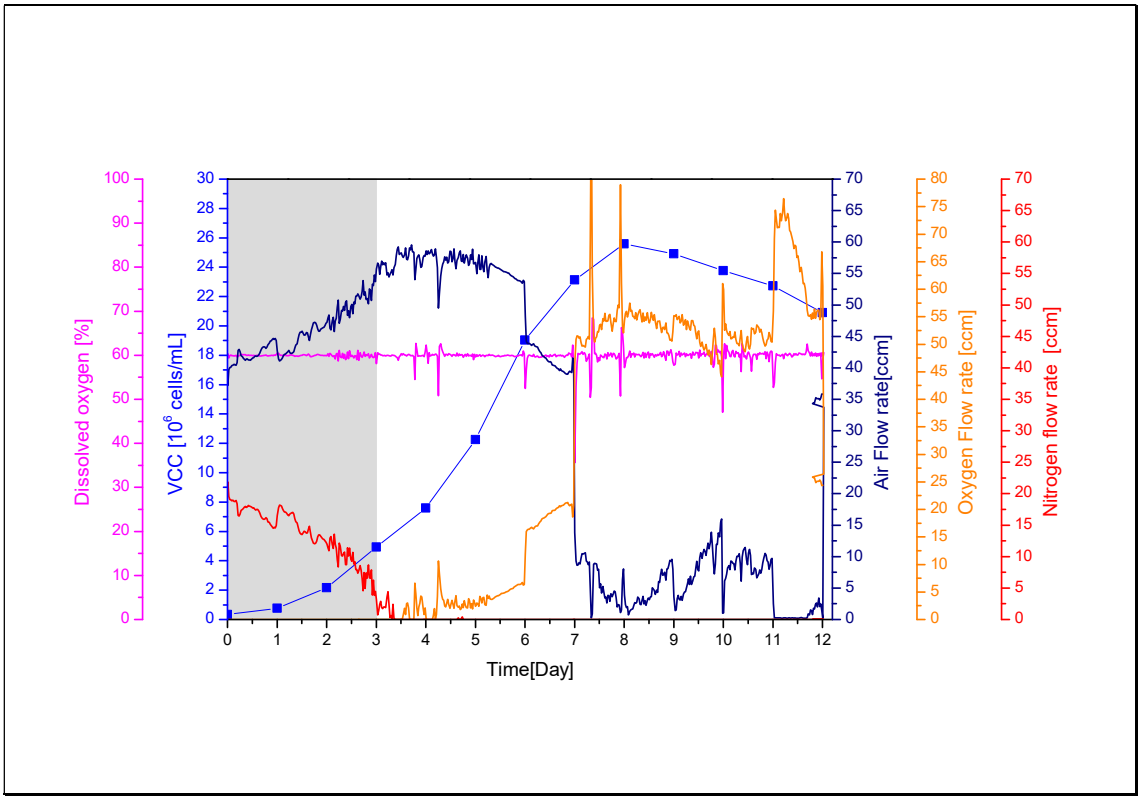


Figure 45: Aeration controlling DO within the 2L glass bioreactor (FBT2_018)

5.7 HPLC for protein analytics

Protein A column was used to estimate the quantity of mAb in the supernatant. The equilibration buffer and elution buffer used were placed in the system at port A and B respectively. Table 28 lists out the formulation of the buffers used for HPLC.

Table 28: Buffers used in Protein A chromatography

Buffers	Component	Concentration	Vendor
Stock solution 1	Monosodium phosphate	78 g/L	Sigma Aldrich, USA
	ddH2O	1000 mL	
Stock solution 2	Disodium phosphate	89 g/L	Sigma Aldrich, USA
	ddH2O	1000 mL	
Equilibration buffer (pH 7.9)	Stock buffer 1	70mL	
	Stock buffer 2	930 mL	
	Sodium chloride	29.22 g/L	Carl-Roth, Germany
Elution buffer (pH 2)	100mM Glycine	7.5 g/L	Carl-Roth, Germany
	Sodium chloride	29.22 g/L	Carl-Roth, Germany
	ddH2O	Upto 1000 mL	
Equilibration buffer and elution buffer is filtered through 0.22µm pore size filter and degassed for 5 minutes			

Before placing the samples in, the entire system is flushed with 15 mL of equilibration buffer. After the system was flushed, the pre-column was first installed and flushed with equilibration buffer at a flow rate of 0.1 mL/min. The column was added to the pre-column and the setup was checked for any leakage. Once the column was installed, the flow rate was increased to 1 mL/min and the system was checked for leakage again. The system was ready to measure the samples once all the initial tests were performed.

Before being placed in the autosampler for measurement, the samples were prepared as per Table 29.

Table 29: Sample preparation

Estimates Protein concentration	Dilution	Volume
0 – 0.5 g/L	1:2	175 µL sample + 175 µL equilibration buffer
0.5 g/L – 2 g/L	1:10	50 µL sample + 450 µL equilibration buffer
> 2 g/L	1:20	25 µL sample + 475 µL equilibration buffer
Note: After sample preparation, the entire volume is filtered through 0.22µm filter and placed in the sampler for measurement.		

The process parameters set in HPLC and the method used for the measurement of protein is listed in Table 30 and Table 31.

Table 30: Process parameter for HPLC

Parameter	**Value**
Flow rate	1 mL/min
Maximum pressure of column	170 bar
Temperature of the sampler	10 °C
Temperature of the oven	30 °C
Time	20 minutes
Injection volume	100µL
UV detector	280 nm

Table 31: HPLC method used for Protein A chromatography

Time [minute]	**Buffer**
0 - 2	Equilibration buffer
2 - 4	Transition from equilibration buffer to elution buffer
4 - 10	Elution buffer
10 - 20	Equilibration buffer

5.8 Reverse Phase- HPLC

Amino acid in the supernatant collected during the entire course of cultivation was measured used RP-HPLC. The reagents used in the process are listed in Table 32.

Table 32: Reagents used in RP-HPLC

Reagent	Component	Quantity	Vendor
Eluent A	Monosodium Phosphate	40 mM	Merck, Germany
	Sodium Azide	5mM	Merck, Germany
Eluent B	Acetonitrile	450 mL	VWR International, USA
	Methanol	450 mL	VWR International, USA
	Water	100 mL	
OPA reagent	o-Phthaldialdehyde	10 mg	Sigma Aldrich, USA
	Mercaptopropionic acid	6.5 µL	Merck, Germany
	Methanol	500 µL	Merck, Germany
	Borate buffer	500 µL	
Borate Buffer (0.4 M, pH 10)	Boric acid	12.36 g	Merck, Germany
	Water	500 mL	
	Sodium hydroxide	For pH adjustment	Merck, Germany
FMOC	9-fluorenyl Methyl Chloroformate	9 mg	Merck, Germany
	Acetonitrile	10 mL	VWR International, USA
Injection diluents for autosampler	Eluent A	100 mL	
	Phosphoric acid	0.4 mL	Sigma Aldrich, USA
Autosampler derivatization	Sample	15 µL	
	OPA	20 µL	
	FMOC	20 µL	
	Injection diluent	50 µL	

Prior to measurement, the cell free samples were diluted with cold methanol and placed in -20 °C overnight, i.e., 100 µL of samples was diluted with 400 µL of cold methanol. The sample was then diluted to 1:50 ratio with borate buffer to increase the pH of the sample (the dilution depended on the estimated quantity of amino acid). The samples were placed in the autosampler and the process parameters used for the measurement is listed in Table 33.

Table 33: Process parameters of RP-HPLC

Parameter	Range
Flow rate	1.5 mL/min
Duration	28 min
Temperature of column oven	40 °C
Fluorescence	Excitation: 330 nm
	Emission: 420 nm
	Excitation: 266 nm
	Emission: 305 nm

The column used for RP-HPLC was Agilent Zorbax Eclipse Plus C18, 3. 5 µm, 4.6 mm x 150 mm and the gradient is listed in Table 34.

Table 34: Gradient used in RP-HPLC for amino acid analysis

Time [min]	Eluent A %	Eluent B %
00.0	96	04
20.0	43	57
20.1	00	100
23.5	00	100
26.6	96	04
28.0	96	04

5.9 Amino acid profile

The cell specific consumption of various amino acids (q_{AA}; where AA is amino acid) was compared against the cell specific productivity (Q_p) and daily growth rate (µ) to study the influence of a particular amino acid on the cell growth of protein production.

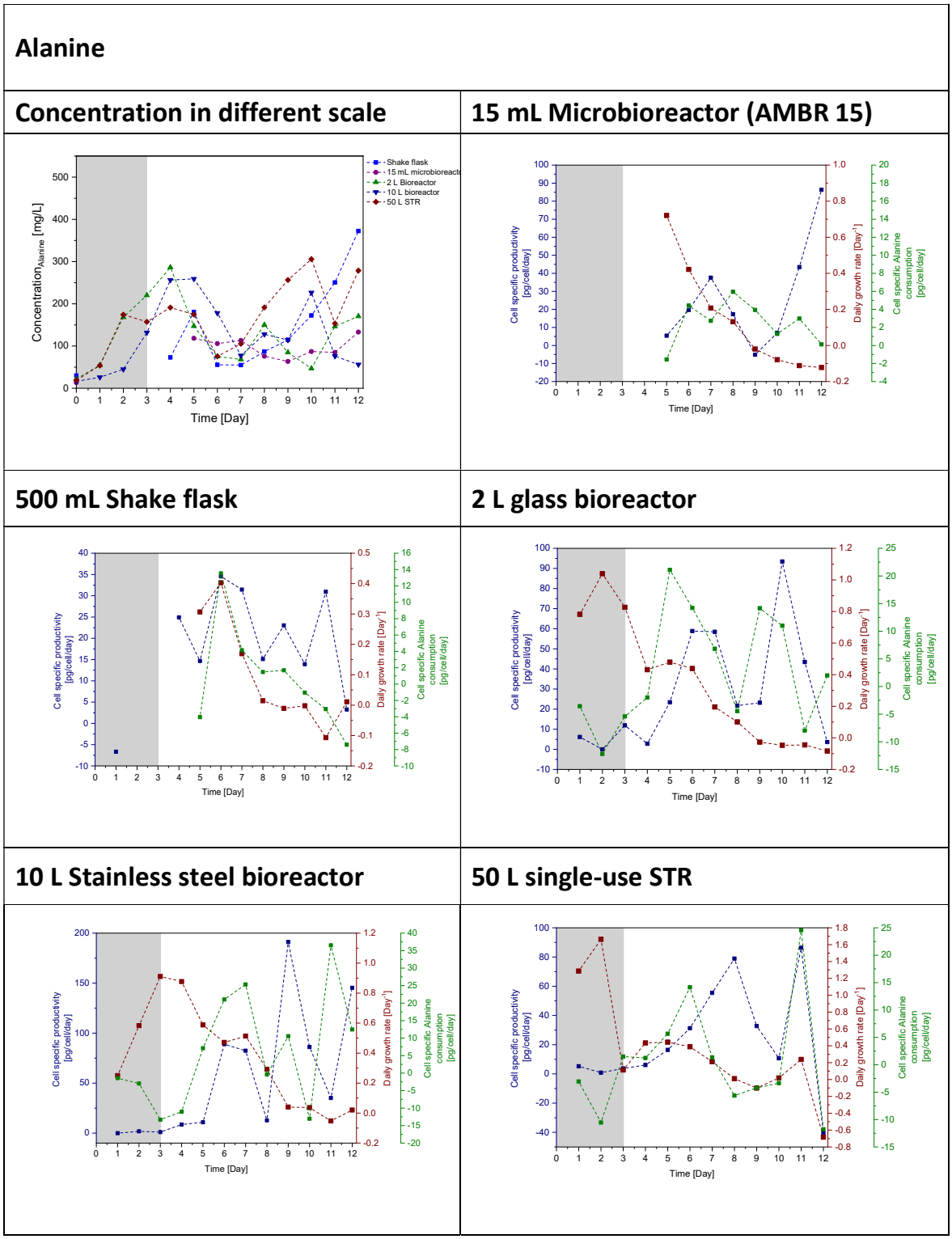


Figure 46 : Study of Alanine in different scales of bioreactor

The production of ammonium and alanine has been linked to the consumption of aspartate and glutamic acids in CHO cell line [90]. The accumulation of alanine in the medium has a negative influence on the cell growth as it inhibits pyruvate kinase and TCA pathway. It is

also a potential source of ammonia [91].

Alanine is formed by trans-amination from pyruvate and is mainly observed in the late stages of the culture. [85][92].

Figure 46 depicts the concentration of alanine in the supernatant during the course of the cultivation and a common pattern observed in the different scales of bioreactor is that the initial concentration of alanine is negligible in the production media, however, the concentration increases during the lag phase of the cell growth and the metabolism switches such that alanine is consumed during the exponential phase and gets accumulated again during the transitioning from exponential phase to stationary phase (i.e., day 6 and 7 onwards).

- 15 mL Microbioreactor (AMBR 15)

Samples were taken day 5 onwards in order to avoid reaching a volume under 10 mL in the bioreactor. The daily growth rate on day 5 reached a maximum value of 0.72 Day^{-1} with a starting cell specific productivity of 5.4 pg/cell/day. The cell specific alanine consumption was the highest on day 8 with a maximum value of 5.95 pg/cell/day and the protein concentration increased exponentially day 9 onwards. The end titre of 3.5 g/L was obtained. No direct correlation between the three components (i.e., growth rate, cell specific productivity and cell specific alanine consumption) was observed in AMBR 15 due to unavailability of samples during the batch phase.

- 500 mL Shake Flask

Shake flask had a starting working volume of 99 mL and the samples were taken on day 0 and day 4 thereafter. The cell specific productivity, growth rate and cell specific alanine consumption was the highest on day 6 with values of 34.55 pg/cell/day, 0.40 day^{-1} and 13.53 pg/cell/day, respectively. The increase in the values corresponded to the switch of growth phase from exponential phase to stationary phase. The consumption of alanine decreased once the cells switched to stationary phase and death phase. The cell specific productivity ranged from 15 to 30 pg/cell/day and no direct correlation between Q_p and q_{Ala} was observed. It indicated the use of alanine by the cells in the earlier growth phases of CHO cell.

- 2 L Glass Bioreactor

The amount of alanine added day 3 onwards to the 2L bioreactor was 75.25 mg per day. The sample was taken everyday to monitor the cell growth and metabolite production and consumption. The maximum growth rate of 1.04 Day $^{-1}$ was reached on day 2. The growth rate during the batch phase ranged from 0.8 to 1.04 Day^{-1}, it was stable in stationary phase with a value of 0.45 Day^{-1}. A maximum cell specific productivity of 58.85 pg/cell/day was observed during the stationary phase and the productivity increased on day 10 with a value of 93.44 pg/cell/day. Alanine consumption also increased to 21.10 pg/cell/day on day 5 during the exponential phase within the cells. An increase in the consumption was also observed on day 9 where the consumption rate increased to 14.17 pg/cell/day. This could be due to a more controlled environment for the growth of cells and the consumption of alanine. It was indicated that alanine could also be used for protein production when the environment in the bioreactors was controlled. This was further investigated in 10 L stainless steel bioreactor and 50 L single-use STR.

- 10 L stainless steel bioreactor

The amount of alanine added to the 10 L bioreactor was 376.27 mg per day during fed batch. During the end of the batch phase, we observed a maximum growth rate of 0.91 Day^{-1}. The cell specific growth rate increased on day 6 during the transition of the cells from exponential phase to stationary phase. A maximum cell specific productivity of 191.14 pg/cell/day was attained on day 9. Alanine consumption increased on day 7 but it reached the maximum value on day 11 with a value of 36.5 pg/cell/day. There was no direct correlation between the three conditions observed in 10 L bioreactor. This could also be attributed to the longer lag phase of the cells in the 10 L reactor.

- 50 L Single-use Stirred tank reactor (STR)

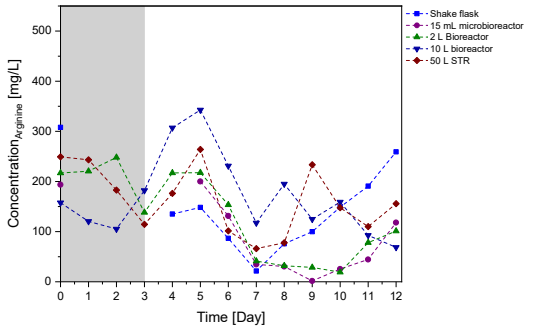
The total amount of alanine added on each day during fed batch was 1881.3 mg. The cell specific productivity gradually increased until day 7 followed by a decrease in productivity. On day 7, the DO reached 0% due to inability to transfer oxygen in the bioreactor for 12 hours. This created a stressful condition for the cells and thereby influencing the productivity. There was sharp increase in the cell specific productivity and alanine on day 11

during the transition of the cells into death phase. A maximum Q_p of 86.34 pg/cell/day and q_{Ala} of 24.57 pg/cell/day was reached on day 11. During the cultivation, a maximum growth rate was measured on day 2 with a value of 1.66 Day^{-1}. At this point the consumption of alanine was minimum and this gradually increased during the exponential phase.

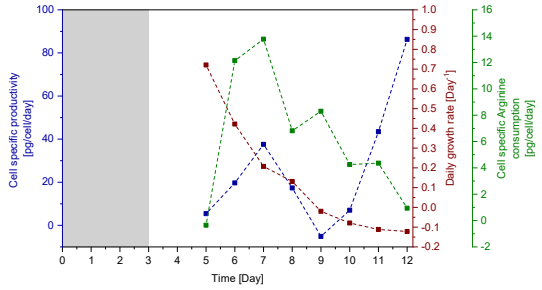
From the 5 different scales it can be conclusively stated that alanine was required for the cell expansion and was mainly consumed in the exponential phase. To optimize the process it is important to supply the medium with sufficient quantity of alanine for the cell growth. Though the percentage of alanine in monoclonal antibody makes up approximately 6 % of the total content, it was not significantly used from the feed medium but the required amount was produced by the cells themselves

Arginine

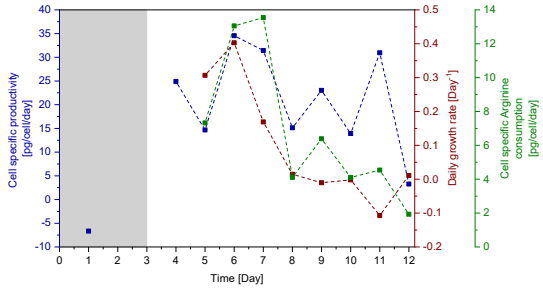
Concentration in different scale



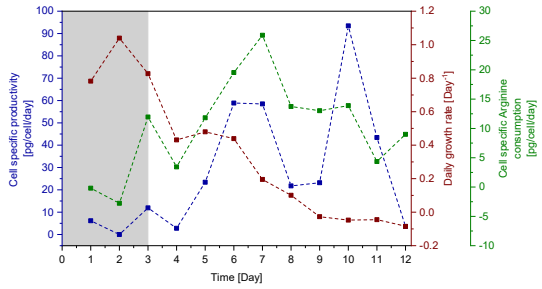
15 mL Microbioreactor



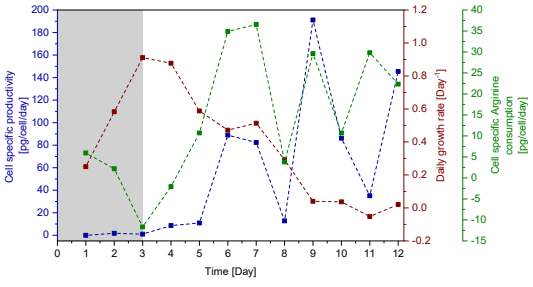
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

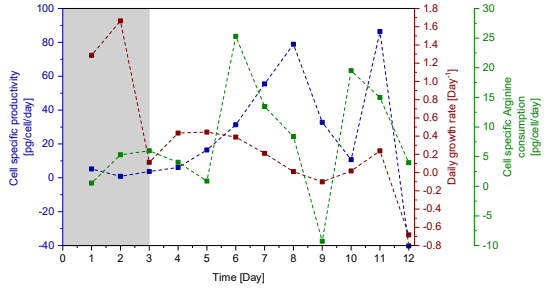


Figure 47 : Study of Arginine in different scales of bioreactor

According to *Torkashvand et. al.*[87], amongst the group of amino acids, arginine has the highest positive effect on the final monoclonal antibody titre response. It is also reported that arginine is important for increasing the performance of mammalian cell culture. [93, 94]

.

An isolated experiment to test the influence of selected amino acid on the mAb titre production and mammalian cell culture performance indicated that arginine, aspartic acid and glutamine had a positive effect on experiments related to their roles in TCA cycle. Arginine, aspartic acid and glutamine entered the TCA cycle through oxaloacetate and α-ketoglutarate, leading to production of more energy and efficient cell metabolism [95].

Gonzalez et. al. [26] used Plackett-Burman statistical design to assess the influence of amino acids on the cell growth and they found that arginine had the highest positive influence on the cell viability.

During the cultivation, arginine concentration in the supernatant of the culture was measured using RP-HPLC and the data from the 5 different scales of bioreactor were depicted in Figure 47.

It was observed that the arginine was consumed until the end of batch phase and was also fed to the bioreactors along with the feed medium. The concentration of the amino acid increased until day 5 and was further metabolised during the stationary phase. The concentration increased again during the death phase.

From the initial assessment of the concentration, it can be stated that arginine was mainly consumed during the stationary phase. This was studied in detail in the following section where the consumption of amino acid and production of protein per cell per day was calculated and compared against growth rate in the cells.

- 15 mL Microbioreactor (AMBR 15)

The amount of arginine added each day to AMBR 15 bioreactors during the fed batch was approximately 1.2 mg per day. The maximum amount of arginine consumed by cells was on day 7 with a value of 13.77 pg/cell/day. Day 8 onwards a reduction in metabolism of arginine was observed. There was no direct correlation between arginine consumption and protein production or growth rate. It can be stated that arginine was used by the cell for production of more energy and other metabolic activities.

- 500 mL Shake Flask

The amount of arginine added to the shake flask day 3 onwards was approximately 11.06 mg per day. On day 6, the maximum consumption of arginine (13.05 pg/cell/day) correlated to

the maximum cell specific productivity (34.55 pg/cell/day) and growth rate (0.4 Day^{-1}). The requirement of arginine reduced day 8 onwards, indicating no direct relation to the production of monoclonal antibody in the shake flask.

- 2 L Glass Bioreactor

The concentration of arginine added to the 2 L bioreactor day 3 onwards was 153.33 mg per day. There was an increase in metabolism when the growth phase shifted from lag to exponential phase. The maximum arginine consumption was measured on day 7 with a value of 25.90 pg/cell/day. The cell specific productivity increased on day 10, however, there was no similar pattern in the consumption of arginine. Indicating that arginine was used for the other metabolic activities apart from cell growth and protein production. This was a sharp contrast to the information obtained from the samples from shake flask. Therefore, these were further studied in the 10 L and 50 L scale bioreactors.

- 10 L stainless steel bioreactor

766.7 mg per day of arginine was added during the fed batch to the 10 L bioreactor. Arginine consumption decreased gradually during the batch phase and it was further metabolised during the exponential phase. A maximum value of 36.56 pg/cell/day was reached on day 7; the cell specific productivity also increased to 82.41 pg/cell/day. On day 9, both the cell specific arginine consumption and cell specific protein production increases with a value of 29.61 pg/cell/day and 191.14 pg/cell/day, respectively. A direct correlation between Q_p and q_{Arg} was observed on day 9.

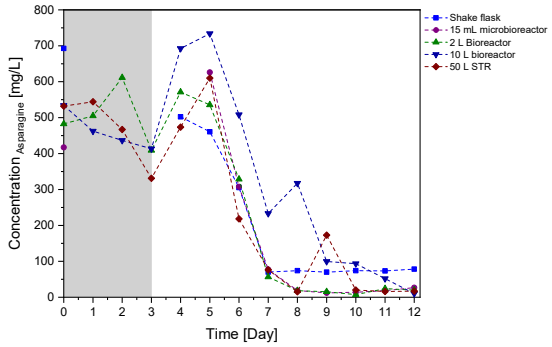
- 50 L Single-use Stirred tank reactor (STR)

The amount of arginine added to the 50 L bioreactor day 3 onwards was 3833 mg per day. Maximum arginine consumption was measured on day 6 with value of 25.29 pg/cell/day and the consumption decreased on the following days. There was no direct correlation between the growth rate, protein production and arginine consumption, indicating the use of arginine for other cellular metabolic activities.

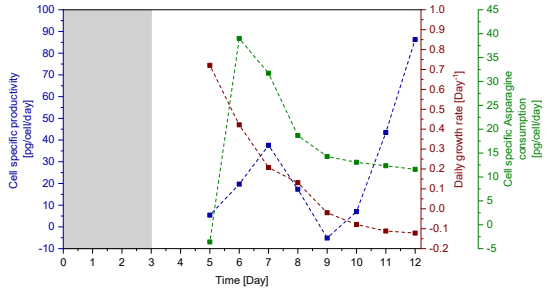
From the 5 different scales, a direct correlation between the three components was not explicitly noted. Indicating the field is open for further testing to study the impact of arginine on the cell growth and protein production.

Asparagine

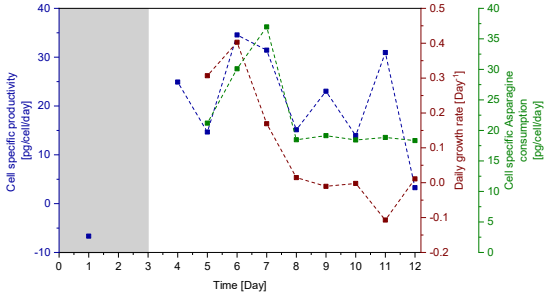
Concentration in different scale



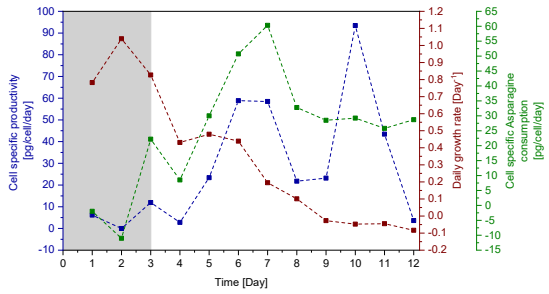
15 mL Microbioreactor (AMBR 15)



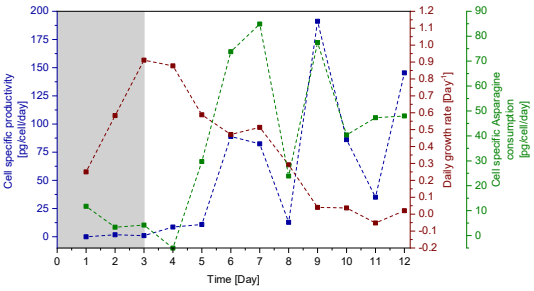
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

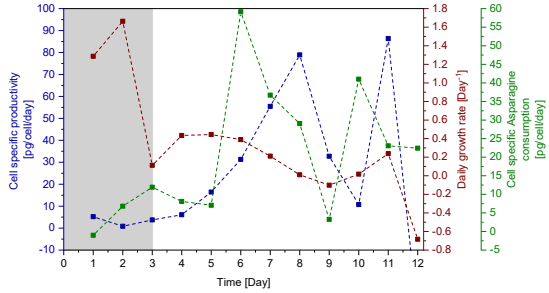


Figure 48 : Study of Asparagine in different scales of bioreactor

High concentration of asparagine has detrimental effect on monoclonal antibody titre and the integral viable cell concentration[87]. It was reported that the addition of asparagine

enhanced the production of ammonium, in turn negatively influence the cell growth at higher concentration [13].

According to *Zhang et. al.* [96] asparagine was important in antibody production phase and the antibody production in CHO-DHFR cell culture could be improved by optimizing the ratio of aspargine to glutamine added to the feed medium.

Asparagine is also essential for the synthesis of glycine, cysteine, proteins, nucleotides and lipids in cell proliferation.

Asparagine can be converted to aspartate through deamination. Furthermore, aspartate uses α-ketoglutarate to form oxaloacetate and glutamate through transamination. The α-ketoglutarate and oxaloacetate can enter TCA cycle and be further metabolized [96].

The concentration of asparagine in the production medium ranged between 400 to 700 mg/L. The CHO cells consumed the amino acid during the batch phase. There was an increase in the concentration during fed batch until day 5 and was metabolised until day 7 and 8. Thereafter, concentration of asparagine was below 100 mg/day until harvest.

The percentage of asparagine in the monoclonal antibody is 4% the total amino acid and the process could be potentially optimized by supplementing asparagine to the bioreactor in two possible methods. One being, supplementing the feed medium with higher concentration of asparagine or feeding the bioreactor with bolus of asparagine. This could further improve the protein production during the cultivation.

- 15 mL Microbioreactor (AMBR 15)

The amount of asparagine added each day to AMBR 15 bioreactors during the fed batch was approximately 2.5 mg per day.

The maximum consumption of asparagine was measured on day 6. During the stationary phase, the consumption remained constant between 11.6 to 14.3 pg/cell/day. The cell specific productivity increased exponentially day 8 onwards, indicating the use of asparagine in protein production in the culture.

- 500 mL Shake Flask

The total amount of asparagine added per day during fed batch in shake flask was 24.5 mg per day. The consumption rate of asparagine was the highest on day 7 with a value of 37

pg/cell/day. The consumption rate was constant day 8 onwards at 20 pg/cell/day and it remained constant until the end of the cultivation, suggesting the use of asparagine in the protein production and use during the stationary phase of the cell growth.

- 2 L Glass Bioreactor

The total amount of asparagine added to the 2 L bioreactor was 340 mg per day during the fed batch. The maximum consumption of asparagine was measured on day 7 with a value of 60.3 pg/cell/day. The consumption rate of asparagine remained constant during the stationary phase of the cells with a rate of approximately 30 pg/cell/day.

- 10 L stainless steel bioreactor

Asparagine was added as a part of FMA and FMB and the total amount added per day during fed batch was 1700 mg per day. The maximum cell specific asparagine consumption was measured on day 7 with a value of 85 pg/cell/day. There were fluctuations in the consumption rate on day 8 and day 9 but this however was constant thereafter. On day 9 the highest cell specific productivity (191.14 pg/cell/day) corresponded to the increase in the metabolism of asparagine (77.5 pg/cell/day), indicating the use of asparagine for monoclonal antibody production.

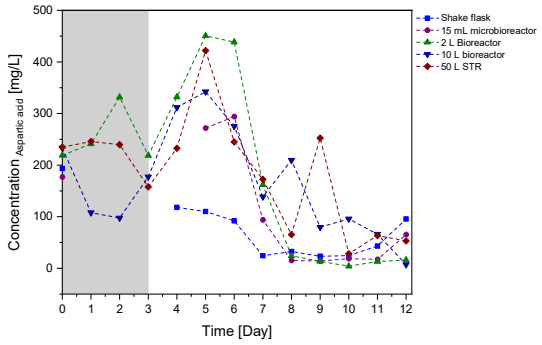
- 50 L Single-use Stirred tank reactor (STR)

The total amount of asparagine added to the 590 L single use bioreactor was approximately 8485 mg per day during the fed batch (day 3 onwards until day 11). The highest consumption rate of asparagine was measured on day 6 with a rate of 60 pg/cell/day. There was sharp decrease in the consumption rate on day 9, this was attributed to switch in metabolism due to the stress caused to the cells as a result of insufficient amount of oxygen supplied to the bioreactor between day 7 and day 8 for 12 hours. There was an increase on day 10 and the consumption remained constant on day 11 and 12. The cell specific productivity increased on day 10 to 86.34 pg/cell/day.

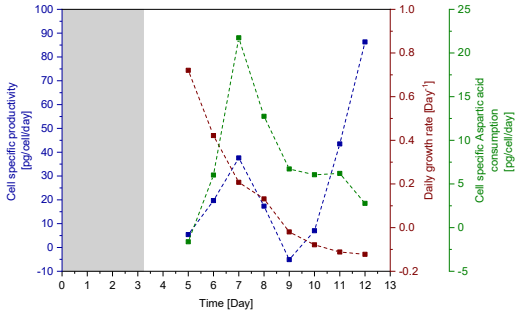
The consumption rate of asparagine was high during the stationary phase and it was inferred that the amino acid was used for the production of monoclonal antibody in CHO DG44 cells. In Figure 48 it was noted that the concentration of asparagine ranged from 0 to 100 mg/L. This could be a bottleneck point for the optimization of the process to increase the protein quality and quantity in the cultivation.

Aspartic acid

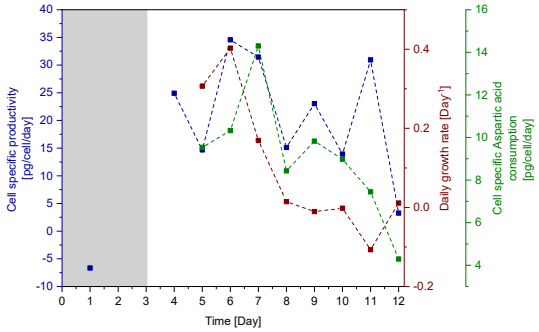
Concentration in different scale



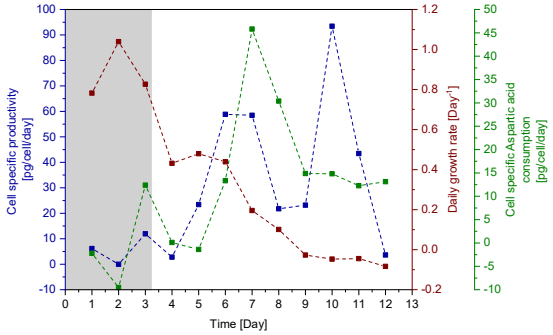
15 mL Microbioreactor (AMBR 15)



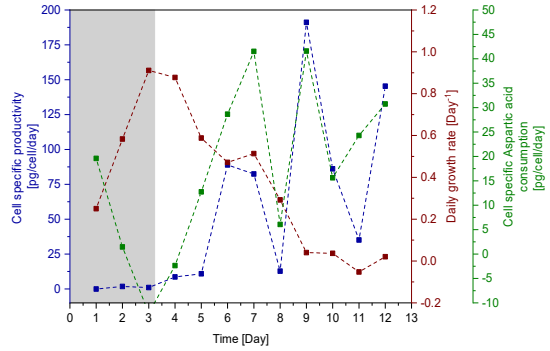
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

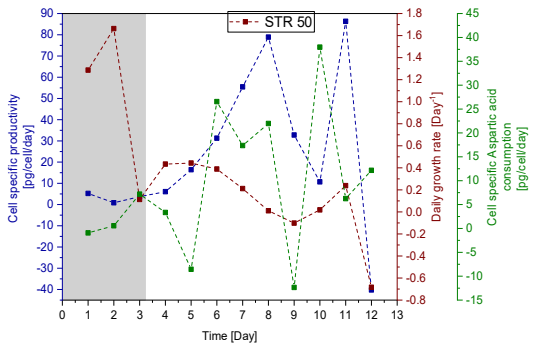


Figure 49 : Study of Aspartic acid in different scales of bioreactor

According to *Torkashvand et. al.*, aspartic acid has high positive effect on the final mAb titre response and IVCC response [87].

Aspartic acid entered the TCA cycle through oxaloacetate and α-ketoglutarate, leading to production of more energy and efficient metabolism in the cells [97]. *William P.K Chong et. al.* demonstrated that aspartic acid (from the medium) contributed to the supply of malate and this led to an increase in the over-expression of malate dehydrogenase II which in turn led to an increase in intracellular ATP and NADH and further improved the integral viable cell count and final mAb titre[98]

Figure 49indicated slow or negligible consumption of aspartic acid during the batch phase and accumulation of the amino acid during the exponential phase. However during the stationary phase, aspartic acid was metabolised, indicating the use of aspartic acid for either protein production or cell functionality during the stationary phase.

- 15 mL Microbioreactor (AMBR 15)

Aspartic acid was added to the bioreactors as a component present in FMA and FMB. The total amount of aspartic acid added to AMBR 15 was 1.19 mg per day during the fed batch until day 11.

The cell specific amino acid consumption and cell specific productivity have a similar pattern until day 9. The maximum cell specific aspartic acid consumption was measured on day 7 with a value of 21.74 pg/cell/day and the cell specific productivity on day 7 was calculated to be 37.6 pg/cell/day. The aspartic acid consumption decreased gradually during the stationary phase and was constantly consumed at the rate of approximately 6 pg/cell/day, indicating the use of aspartic acid in production of the desired protein.

- 500 mL Shake Flask

The total amount of aspartic acid to the 500 mL shake flask during fed batch was 11.8 mg per day until day 11. The cell specific aspartic acid consumption was measured and the maximum consumption was observed on day 7 with a value of 14.3 pg/cell/day. The cell specific amino acid consumption and cell specific productivity follow a similar pattern with a high cell specific productivity on day 6/7 and an increase in value on day 11. Cell specific aspartic acid consumption during the stationary phase gradually decreases.

- 2 L Glass Bioreactor

The amount of aspartic acid added to the 2L bioreactor during fed batch was measured to be 163.5 mg per day. The maximum consumption of the amino acid was measured on day 7 with a value of 45.81 pg/cell/day. The metabolism of aspartic acid decreased until day 9. The consumption rate of aspartic acid was maintained at a constant range of 12.3 pg/cell/day to 15 pg/cell/day until the end of cultivation. The cell specific productivity also increased in day 5 and 6 and reached a maximum value of 93.43 pg/cell/day on day 10.

- 10 L stainless steel bioreactor

The amount of aspartic acid added to the 10 L bioreactor daily was 818 mg and this was added until day 11 of the cultivation. The consumption rate of aspartic acid decreased gradually during the batch phase and metabolism increased during the fed batch until day 7 with a highest value measured of 41.5 pg/cell/day. On day 9, an increase in both cell specific aspartic acid consumption and cell specific productivity was observed, indicating the use of aspartic acid for the production of monoclonal antibody.

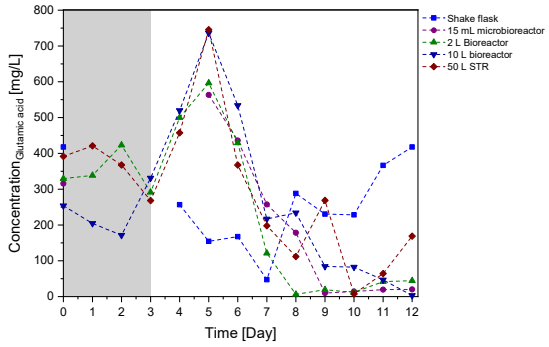
- 50 L Single-use Stirred tank reactor (STR)

Aspartic acid amount fed to the 50 L bioreactor was measured to be 4090 mg per day from day 3 to day 11. This was consumed by the cells for cell growth and protein production. The maximum consumption of aspartic acid was measured on day 10 with a value of 38 pg/cell/day. The cell specific productivity was the highest on day 8 and day 11 with 79 pg/cell/day and 86.34 pg/cell/day.

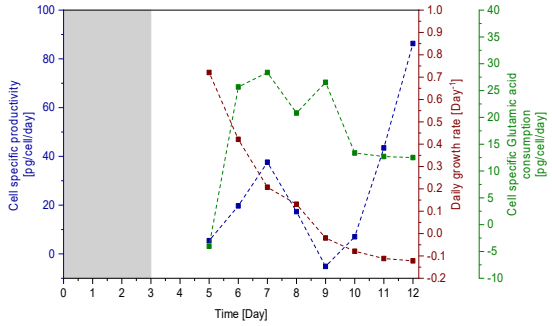
From the different bioreactors, it can be concluded that the consumption of aspartic acid was at its peak during transition from exponential phase to stationary phase. During the stationary phase, the consumption in most bioreactor remained constant indicating the use of aspartic acid for both cell growth and protein production. The graph with the compiled cumulative concentration in the bioreactors depicted a depletion day 8 onwards. This was a potential bottleneck and the concentration of aspartic acid can be increased in the feed medium (especially after day 7) to increase the product concentration in the cultivation

Glutamic acid

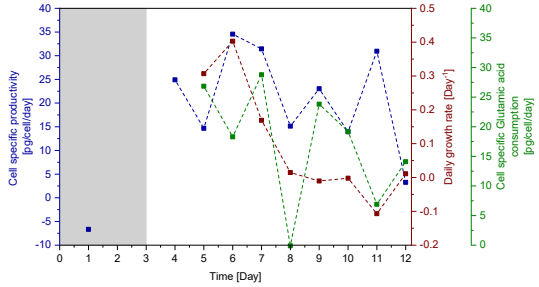
Concentration in different scale



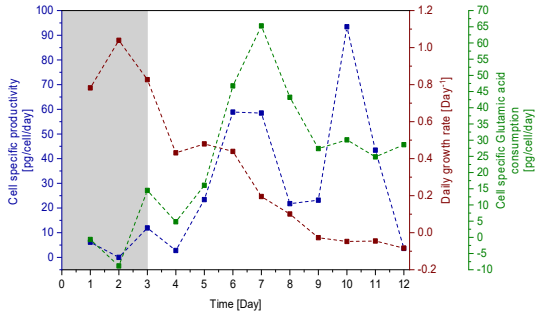
15 ml Microbioreactor (AMBR 15)



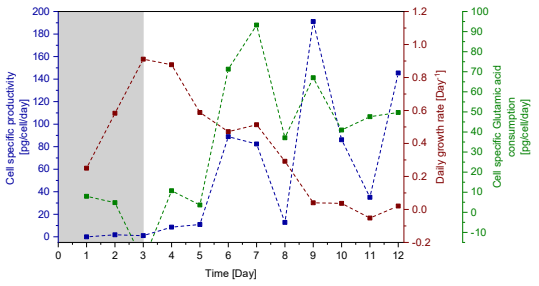
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

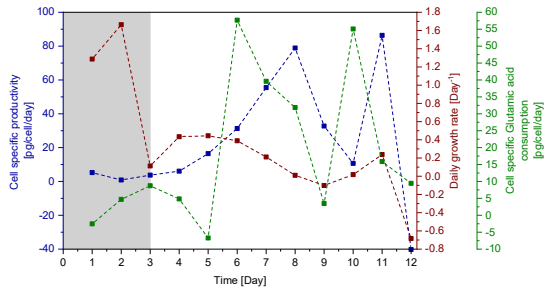


Figure 50: Study of Glutamic acid in different scales of bioreactor

Glutamic acid has a positive impact on the final mAb titre and viable cell concentration according to the examination by *Torkashvand et. al.* [87]. Glutamic acid along with asparagine and aspartic acid enters the TCA cycle through oxaloacetate and α-ketoglutarate,

which leads to an increase in energy and efficient cell metabolism [97].

Another important component synthesized using glutamic acid and glycine is glutathione. This is a well-known indicator of oxidative stress [99]. High level of glutathione has been related to high productivity [99].

In aerobic cells, mitochondrial respiration is the main cause of reactive oxygen species (ROS) [100]. It has been hypothesized by *Torkashvand et. al.* that high monoclonal antibody producers experience increased aerobic metabolism and in turn results in greater generation of ROS [87]. Stress is a possible reason for cellular apoptosis and growth limitation in fed batch culture. The ideal condition would be to decrease stress and amount of glutathione thereby decrease the negative effect on cell growth and in turn enhance culture performance and productivity [101]. Since glutamic acid and glycine are closely linked to glutathione, the positive effect of these amino acids is related to their role in glutathione pathway [99].

The effect of glutamic acid and glycine are cell line and clone specific. The data is compared for the clone in use and the influence of glutamic acid on cell growth and protein production is compared in the five scales of bioreactor.

Figure 50depicted the glutamic acid concentration in the supernatant collected every day of the cultivation in the five different scales of bioreactor. The initial concentration of glutamic acid in the production medium ranged from 250 mg/L to 400 mg/L. The concentration of glutamic acid remained constant during the batch phase and an increase in metabolism was observed under the exponential. Accumulation in teh supernatant occurred until day 5 and was metabolised during the transition from exponential phase to stationary phase. The cell specific glutamic acid consumption was compared to the cell growth and cell specific productivity to establish the connection between the three parameters.

- 15 mL Microbioreactor (AMBR 15)

Glutamic acid is known to have a positive impact on the IVCC and the final mAb titre response. These are specific to the strain of CHO and this was measured in the cell line used to understand the influence of the amino acid on these parameters. To evaluate this, the cell specific consumption rate, growth rate and cell specific productivity was studied for all the bioreactors. For AMBR 15, the total amount of glutamic acid added every day during the fed batch was 2.54 mg per day. The consumption of glutamic acid ranged from 20 pg/cell/day to 30 pg/cell/day. Towards the end of the cultivation, the consumption remains constant at 13

pg/cell/day. The cell specific productivity increased from day 5 to day 7 and the production of monoclonal antibody decreased until day 9, followed by constant increase until day 12. To understand the influence, more data point would be needed to explain the correlation between the three entities.

- 500 mL Shake Flask

The amount of glutamic acid added per day was measured to be 25.32 mg per day during the fed batch. The maximum glutamic acid consumed was measured on day 7 with a value of 28.78 pg/cell/day. The productivity and consumption of glutamic acid also increases on day 9 when the cells were in the stationary phase. The productivity during the stationary phase was the highest on day 11, however there was no direct correlation between the amino acid used and protein produced in this phase.

- 2 L Glass Bioreactor

The amount of glutamic acid added to the 2 L bioreactor was measured to be 350.89 mg per day during the fed batch. The consumption of glutamic acid was slow during the batch phase and it gradually increased until day 7 with a maximum value of 65.26 pg/cell/day. The rate decreased until day 9 and was constant between the range of 25 pg/cell/day to 30 pg/cell/day. The cell specific productivity was maximum on day 10, however, there was no trend in the consumption of amino acid and productivity.

- 10 L stainless steel bioreactor

Feed medium added per day to the 10 L reactor during the fed batch contained 1754.43 mg of glutamic acid. The consumption of glutamic acid was slow during the batch phase and gradually increased to a maximum value of 93.27 pg/cell/day on day 7. The rate remained constant until the end of the cultivation indicating the constant consumption for normal functionality of the cells in the bioreactor. The productivity is reached its peak on day 9 with a value of 191.14 pg/cell/day and the corresponding cell specific glutamic acid consumption was measured to be 67 pg/cell/day.

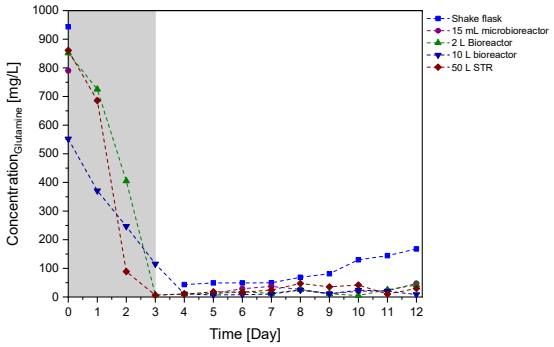
- 50 L Single-use Stirred tank reactor (STR)

The amount of glutamic acid added during the fed batch was measured to be 8772 mg per day. The consumption increased on day 6 (during cell transition from exponential phase to stationary phase) and on day 10 (during cell transition from stationary phase to death phase). The value on day 6 was measured to be 58 pg/cell/day and the value on day 10 was 55 pg/cell/day. The three entities were not correlated in the large scale. A deviation can also be contributed due to the stress caused in the cells on day 7 as a result of oxygen depletion for 12 hours.

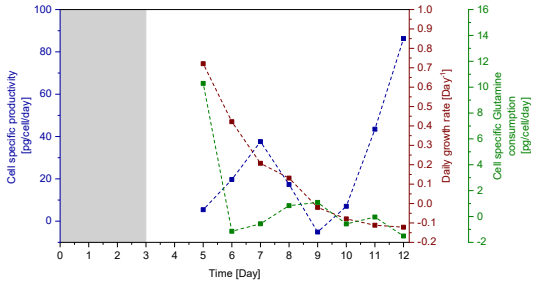
The concentration of glutamic acid increased in the cell culture medium until day 5 and was metabolised by the cells during the transition from exponential phase to stationary phase. The concentration was below 100 mg/L in all the reactors excluding 150 mL shake flask. The cells constantly consumed glutamic acid till the end of the cultivation. The amount of glutamic acid can be increased to positively impact the cell growth during the stationary phase of the growth phase.

Glutamine

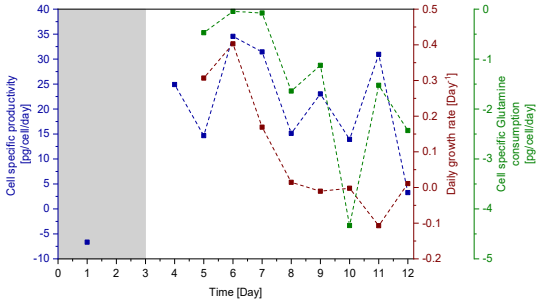
Concentration in different scale



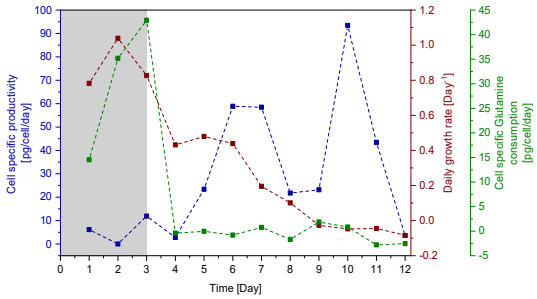
15 mL Microbioreactor (AMBR 15)



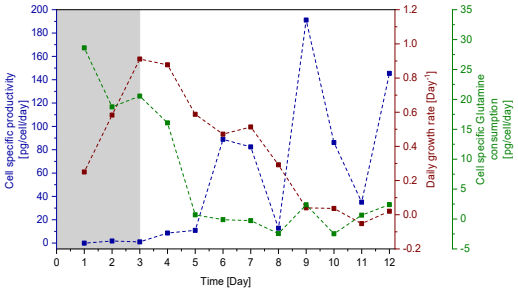
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

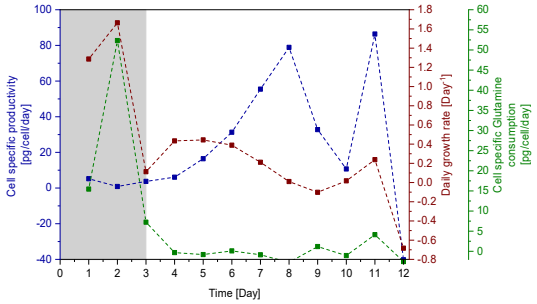


Figure 51 : Study of Glutamine in different scales of bioreactor

Glutamine is metabolised and depleted when the cells are in the G1 phase and the cells continue to traverse to S, G2 and M phase. Depletion of glutamine and isoleucine in the medium results in inability of cells to proliferate further to the other phases, thereby

stagnating the growth of the cells to G1 phase. It continues to remain in the G1 phase until further supplemented with glutamine or isoleucine [102].

Figure 51depicted the concentration of glutamine in the medium during the cultivation. The maximum concentration was metabolised during the batch phase and since the concentration of glutamine in the feed medium was negligible, there was no significant increase in the amount of glutamine. However, an increase in the concentration of glutamine is observed during the end of the cultivation caused due to conversion of glutamate to glutamine as discussed in Section 3.

- 15 mL Microbioreactor (AMBR 15)

The amount of glutamine added during fed batch was 0.0057 mg per day during fed batch. The cell specific consumption of glutamine was expected to be higher during batch phase, however, the data was missing due to no sampling until day 5.

- 500 mL Shake Flask

The amount of glutamine present in feed medium supplied day 3 onwards was 0.057 mg per day. The glutamine consumption was negligible day 5 onwards and the data was insufficient to study the impact of the amino acid on the cell growth or protein production.

- 2 L Glass Bioreactor

Samples were taken everyday during the culture run in the 2 L bioreactor. Glutamine concentration was measured and the cell specific glutamine consumption calculated based on the data. As depicted in Figure 51, the glutamine consumption rate increased until day 3 followed by drastic drop to 0 pg/cell/day. The cell specific glutamine consumption reached a maximum of 43 pg/cell/day. This indicated the use of glutamine for the initial growth of the cells.

- 10 L stainless steel bioreactor

The total amount of glutamine added to the 10L reactor was 3.96 mg per day during the fed batch until day 11. The maximum cell specific consumption was observed on the first day

with a value of 28.6 pg/cell/day followed by gradual decrease in the consumption until day 5. From prehistoric data for the 10L reactor, a longer lag phase was observed and this coincided with the decrease in consumption of glutamine.

- 50 L Single-use Stirred tank reactor (STR)

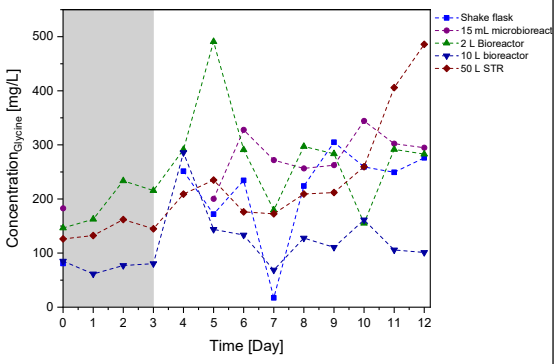
The concentration of glutamine fed to the bioreactor daily was 10.79 mg per day from day 3 to day 11. The maximum cell specific consumption of glutamine was observed on day 2 with a value of 52.33 pg/cell/day with a maximum growth rate of 1.66 Day^{-1}. The maximum consumption of amino acid occurred during the batch phase.

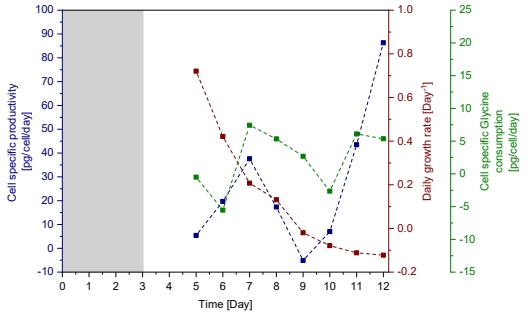
From the data presented above and the prior knowledge of use of glutamine in cell proliferation, it was concluded that the glutamine was extensively used by the cells for cell growth. For further studies, the concentration of glutamine can be increased or a bolus feed can be added to the cell culture until the cells reach the stationary phase, thereby increasing the cell concentration of the culture.

Glycine

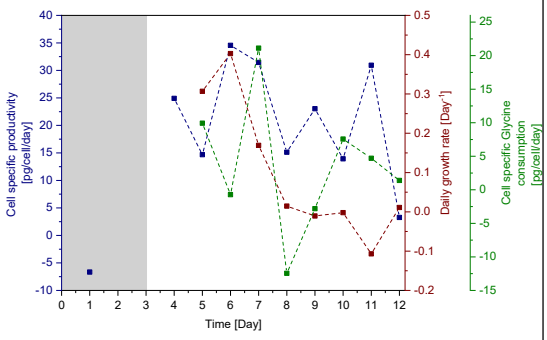
Concentration in different scale

15 mL Microbioreactor (AMBR 15)

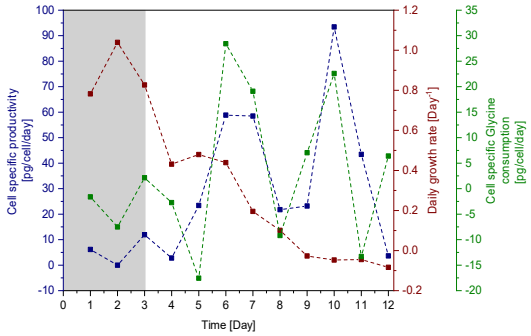




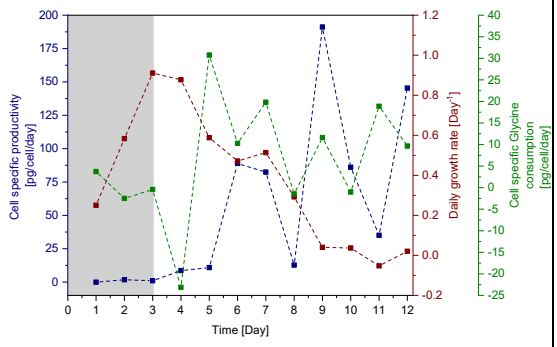
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

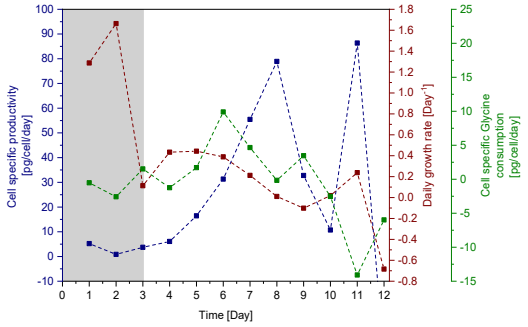


Figure 52: Study of Glycine in different scales of bioreactor

Addition of glycine is known to improve the CHO cell growth and recombinant protein levels.

Metabolic parameters such as glutamine utilization, final ammonium levels, glucose consumption and lactate production are positively impacted by addition of glycine. Further, it can also be used to mitigate toxic effects of ammonium on cell growth protein production and quality [93]. Experiments conducted by *Chen et. al.* concluded that the addition of glycine improved the final cell density by 25% [13]

Glycine is also readily converted to threonine. The mechanism of degradation of glycine and threonine increases the pyruvate levels and results in an increased flux through TCA cycle, which in turn improves cell energetic.

Figure 52represented the amount of glycine present in the supernatant during the entire cultivation of 12 days. The concentration ranged between 100 to 300 mg/L in the different scales indicating presence of sufficient amount in the medium during the entire course of cultivation.

- 15 mL Microbioreactor (AMBR 15)

The amount of glycine present in the feed medium added daily was measured to be 0.57 mg per day. The consumption of glycine was comparatively lower to the other amino acids and the maximum consumption was measured on day 7 with a value of 7.43 pg/cell/day. The corresponding cell specific productivity on day 7 was measured to be 37.6 pg/cell/day. Productivity increases towards the end of the process and the rate of consumption of glycine was also maintained at 5 pg/cell/day to 6 pg/cell/day, suggesting the use of glycine for protein production apart from normal functionalities of the cell culture.

- 500 mL Shake Flask

The total amount of glycine added to the shake flask was 5.69 mg per day during the fed batch. The maximum consumption of glycine was observed on day 7 with a value of 21.12 pg/cell/day and a productivity of 31.4 pg/cell/day. Glycine was also consumed during the end of the cultivation and this signified the use of this amino acid in protein production.

- 2 L Glass Bioreactor

In 2 L bioreactor, the amount of glycine added during the fed batch was 78.86 mg per day. The cell specific glycine consumption increased on day 6 and day 10 with values 28.42

pg/cell/day and 22.58 pg/cell/day respectively. The consumption pattern corresponded to the cell specific productivity.

- 10 L stainless steel bioreactor

The amount of glycine added to the 10 L reactor via the feed medium was measured to be 394.32 mg per day from day 3 to day 11. The lag phase in the 10 L bioreactor was longer than the other bioreactors due to high oxygen concentration in the surrounding. This, however, did not influence the consumption of glycine during the entire course of the cultivation. The maximum cell specific glycine consumption was noted on day 5 with a value of 30.76 pg/cell/day and was constantly metabolised by the cells till the end of cultivation.

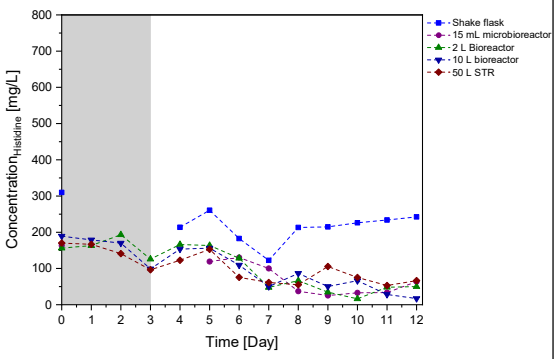
- 50 L Single-use Stirred tank reactor (STR)

Glycine was added to the bioreactor through the feed medium and the total amount added per day was 1971.30 mg per day. The maximum consumption of glycine occurred on day 6 with a value of 9.9 pg/cell/day. The consumption of glycine reduced at the end of the cultivation. This could be due to the stress caused by insufficient oxygen supplied to the cells on day 7.

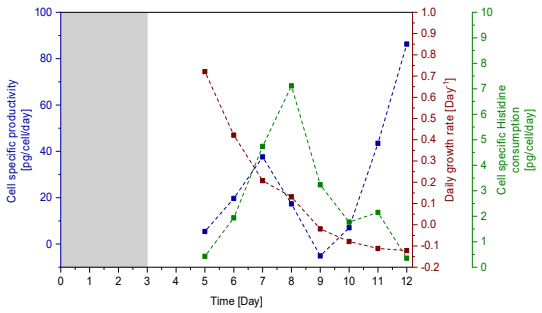
Glycine is known to have a positive impact on the cell growth and protein production and the impact was studied in the different scale of bioreactors. During the comparison of the glycine consumption in the different scales, the use of glycine for cell growth and protein production was evident. Sufficient amount of glycine was present throughout the duration of the cultivation, therefore the focus can be shifted from glycine to the other amino acids for the optimization of the process.

Histidine

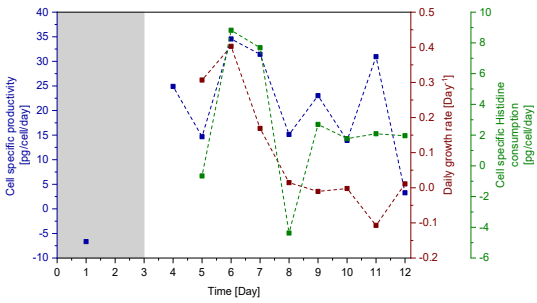
Concentration in different scale



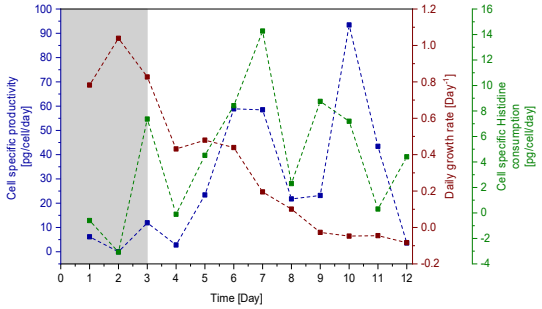
15 mL Microbioreactor (AMBR 15)



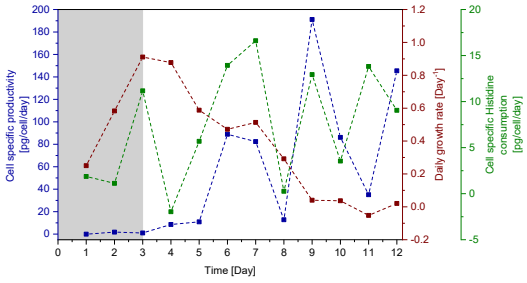
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

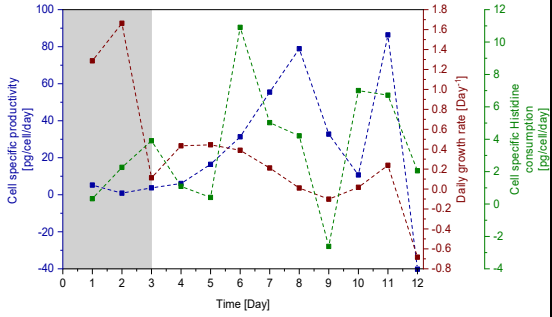


Figure 53 : Study of Histidine in different scales of bioreactor

Carrillo-Cocom et. al., established that more than 90% of histidine present in the medium is consumed during the batch phase. Even though the histidine undergoes maximum conversion in both naïve and recombinant cells, its use in production of recombinant protein

is the least when compared to the other amino acids. [103] .

During the fed batch in this process, approximately 1065 mg/L of histidine was present in the feed medium, of which a certain fraction is supplemented to the cell culture in the different scales of bioreactor. The amount of histidine was measured in the supernatant and the results were represented inFigure 53 . The concentration of histidine decreased from 200 mg/L to 100 mg/L during batch phase and it was accumulated during exponential phase and further metabolised day 5 onwards. The detail of cell specific consumption with respect to different scale was enlisted in the subtopic.

- 15 mL Microbioreactor (AMBR 15)

AMBR 15 was added with 0.43 mg of histidine daily during the fed batch phase. The consumption of histidine was at peak on day 8, however, the consumption rate was lower when compared to the other amino acids used for the process. The maximum consumption on day 8 was measured to be 7.12 pg/cell/day.

- 500 mL Shake Flask

The amount of histidine added to shake flask during fed batch was 4.24 mg per day. The cell specific histidine consumption was at a peak on day 6 and 7 with a value of 8.82 pg/cell/day and 7.68 pg/cell/day respectively. A similar pattern was observed with respect to the cell specific productivity, with the peak observed on day 6 and day 11 (day of transitions between different growth phases) with a value of 34.55 pg/cell/day and 30.93 pg/cell/day.

- 2 L Glass Bioreactor

The amount added in the 2 L reactor was calculated to be 58.70 mg per day during fed batch in the bioreactor. The maximum histidine was consumed on day 7 with a value of 14.27 pg/cell/day. The consumption declined at the end of the process, but was constant consumed indicating the use of histidine for metabolic activities of the cell.

- 10 L stainless steel bioreactor

10L bioreactor was fed with 293.51 mg of histidine per day during the fed batch process. The cell specific consumption was elevated on day 7, day 9 and day 11 with 16.61 pg/cell/day, 12.92 pg/cell/day and 13.81 pg/cell/day respectively. The cell specific protein production was at a peak on day 9 with a value of 191.14 pg/cell/day. Histidine is constant consumed to perform normal metabolic functions within the cell.

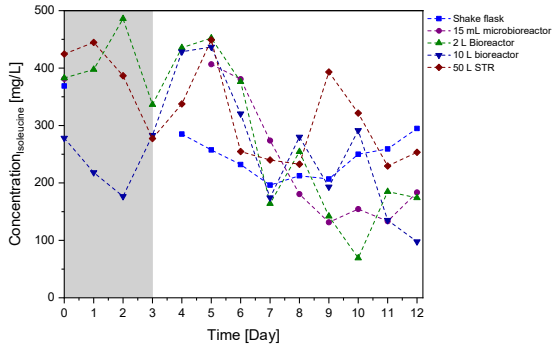
- 50 L Single-use Stirred tank reactor (STR)

Histidine added to the 50 L reactor was measured to be 1467.53 mg per day during the fed batch phase. The peak of histidine consumption was observed on day 6 with a value of 10.9 pg/cell/day. A drop was observed on day 9 and this was due to the stress caused on the cells as a result of oxygen depletion in the system for 12 hours between day 7 and day 8. The cells recovered and consumed histidine on day 10 and 11.

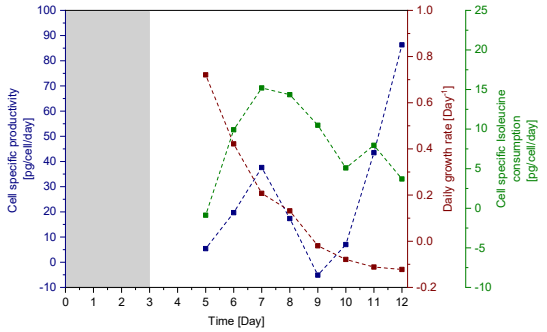
Contrary to studies conducted by *Carrillo-Cocom et. al.,* histidine was constantly consumed throughout the cultivation and was used for the daily functionality of the cell culture. The concentration of histidine ranged between 0 to 100 mg/L in the medium and this could be further studied by addition of bolus histidine solution.

Isoleucine

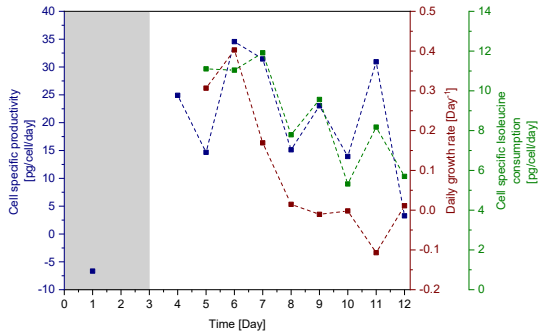
Concentration in different scale



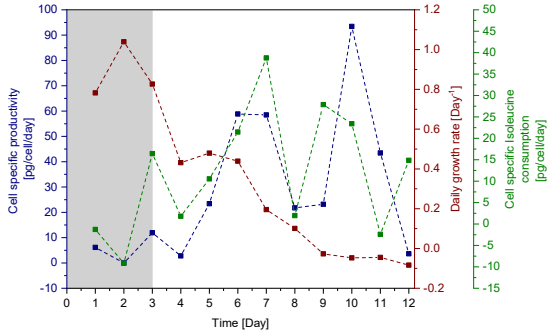
15 mL Microbioreactor (AMBR 15)



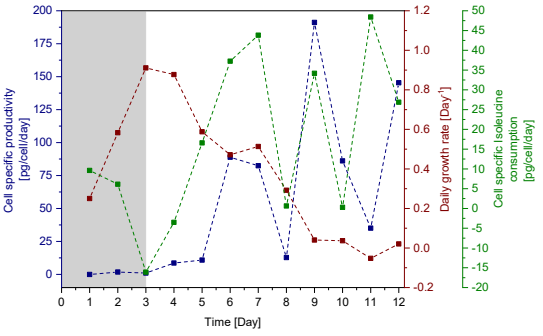
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless Steel bioreactor



50 L Single-use STR

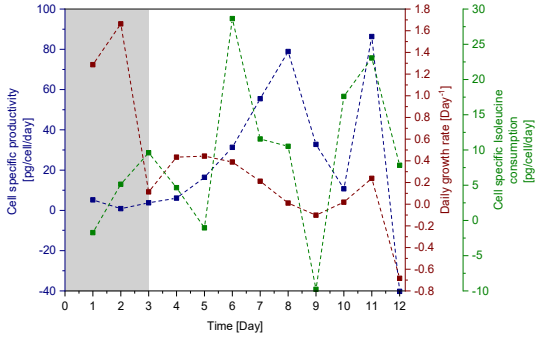


Figure 54 : Study of Isoleucine in different scales of bioreactor

Tobey and Ley from biomedical research group of University of California tested the growth of CHO cells in isoleucine deficient medium supplemented with dialyzed sera. The entire

population of cells were arrested in G1 state within 24 to 36 hour. Addition of isoleucine initiated DNA synthesis and further continued the cell division [102]. Indicating isoleucine is mainly used for cell growth and expansion.

The percentage of isoleucine in the monoclonal antibody was measured to be only 2.26 % the total content. However, the concentration of isoleucine in feed medium was 3172 mg/L. A calculated percentage of isoleucine was added to the bioreactor based on the amount of feed medium added based on the scale of the bioreactor. Figure 54represented the amount of isoleucine present in the medium during the 12 day course of cultivation. The amino acid was metabolised during the batch phase and was accumulated until day 5 after which it was gradually consumed until day 7 and the quantity in the medium remained constant during the stationary and death phase.

- 15 mL Microbioreactor (AMBR 15)

Isoleucine is used for the growth of the cells and the amount added to the bioreactor via feed medium was 1.24 mg of isoleucine per day. The data was available from day 5 onwards and the peak was reached on day 7 with a value of 15.2 pg/cell/day. After day 7 the consumption reduces gradually, but, was constantly consumed till the end of cultivation.

- 500 mL Shake Flask

The amount of isoleucine added during fed batch was 12.33 mg per day until day 11. The consumption was at peak on day 7 with a value of 11.9 pg/cell/day. The consumption declined gradually till the end of the process indicating the use of isoleucine for growth, protein production and normal functionality of cells during the cultivation.

- 2 L Glass Bioreactor

The amount of isoleucine added in the 2 L BDCU was 170.85 mg per day. The peak consumption was measured on day 7 with a value of 38.77 pg/cell/day. The cells consume isoleucine till the end of cultivation.

- 10 L stainless steel bioreactor

Isoleucine added to the bioreactor through feed medium was 854.28 mg per day. The consumption reached its peak on day 7, day 9 and day 11 with values of 43.8 pg/cell/day, 34.18 pg/cell/day and 48.42 pg/cell/day, respectively. The maximum cell productivity was observed on day 9 with a value of 191.14 pg/cell/day. From the Figure 54 it can be estimated that the amino acid was used for cell growth in initial growth phase and was also used by cells for protein production .

- 50 L Single-use Stirred tank reactor (STR)

Isoleucine added in 50 L bioreactor was a total of 4271.32 mg per day during fed batch phase. The peak was observed on day 6 and day 11 with values of 28.65 pg/cell/day and 23.05 pg/cell/day. Isoleucine was consumed for the protein production during the end of the cultivation when the cells transition from stationary phase to death phase.

Isoleucine was constantly consumed by the cells in all the 5 different scale of bioreactors, indicating the use if this amino acid for cell growth and proliferation during the entire duration of cultivation. The maximum consumption of isoleucine occurred during the transition of the cells from exponential phase to stationary phase (on day 7). The presence of isoleucine ensured the successful growth of cells within the sterile environment of a bioreactor. However, the quantity of isoleucine is sufficiently available in the culture medium and the focus of the optimization of medium can be switched to other amino acids.

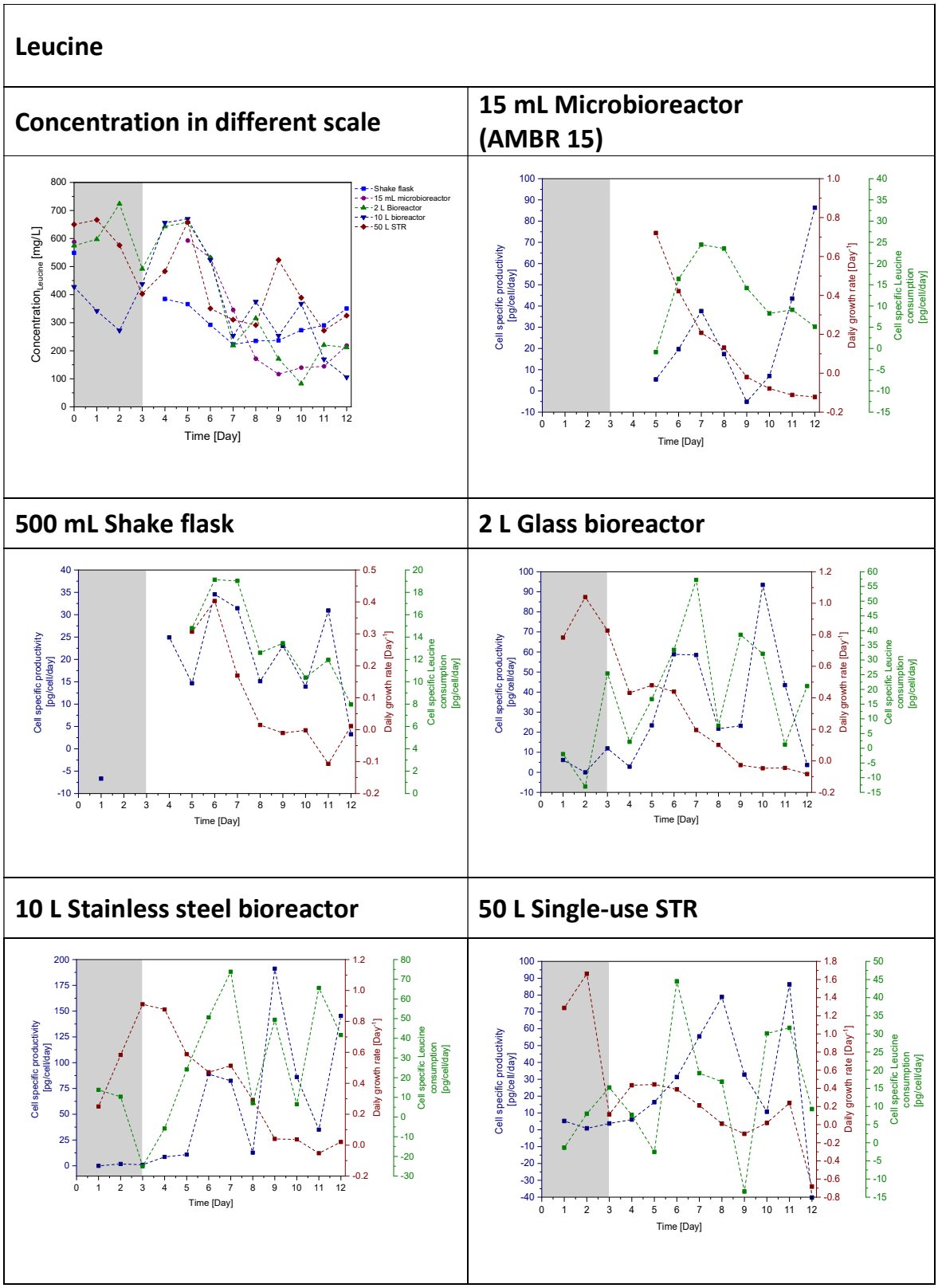


Figure 55 : Study of Leucine in different scales of bioreactor

Gonzalez et. al. used plackett-burman statistical design to study the effect of amino acid on cell growth and protein production. They found leucine had highest negative impact on cell

growth and viability. [26]

It was vital to maintain the low amount of leucine to ensure transport of other amino acids in the cells [104].

The amount of leucine in the production medium ranged from 400 to 650 mg/L which was consumed during the batch phase and leucine was accumulated in the medium during the exponential phase. It was metabolised during transition from exponential to stationary phase and was maintained at constant concentration day 7/ 8 onwards. The amount of leucine in feed was measured to be 4600 mg/L and a certain percentage of this was supplemented to the respective bioreactors. The cell specific leucine consumption was measured for the 5 different scales of bioreactor and the data was compared to the cell specific productivity and cell growth.

- 15 mL Microbioreactor (AMBR 15)

The pattern followed in leucine was similar to isoleucine. The amount of leucine added to AMBR 15 bioreactor during fed batch was 1.83 mg per day. The maximum amount consumed was on day 7 with a value of 24.47 pg/cell/day. The cell specific productivity also increased on day 7 with a value of 37.6 pg/cell/day.

- 500 mL Shake Flask

The amount of leucine added to the shake flask was 18.21 mg per day. The peak value was measured on day 6 and 7 with a value of 19.13 pg/cell/day and 19.02 pg/cell/day respectively. The consumption gradually declined, however, it was constantly consumed by the cells till the end of the cultivation. On day 11, the peak cell specific productivity of 30.39 pg/cell/day was reached and the corresponding leucine consumption was measured at 11.96 pg/cell. A direct correlation between the leucine metabolism and protein production was observed.

- 2 L Glass Bioreactor

The amount of leucine added to the 2 L BDCU bioreactor was 252.39 mg per day during the fed batch until day 11. The elevation in the consumption was noted on day 3, day 7 and day 9 with a value of 25.4 pg/cell/day, 57.24 pg/cell/day and 38.57 pg/cell/day, respectively. The

cells constantly consumed leucine till the end of the cultivation.

- 10 L stainless steel bioreactor

Leucine added to the bioreactor during fed batch was measured to be 1261.93 mg per day. The peak of consumption was observed on day 7, day 9 and day 11 with value of 73.82 pg/cell/day, 49.4 pg/cell/day and 65.6 pg/cell/day. On day 9, the cell specific productivity was at a peak and consumption of leucine also peaked during the respective day. Thereby indicating a correlation between the two parameters.

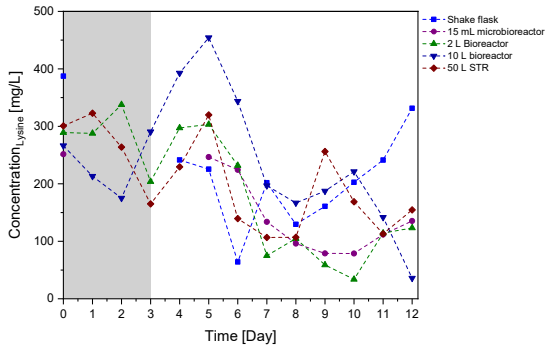
- 50 L Single-use Stirred tank reactor (STR)

Leucine added in 50 L bioreactor was a total of 6310 mg per day during fed batch phase. The peak was observed on day 6 and day 11 with values of 44.5 pg/cell/day and 31.67 pg/cell/day. Leucine was consumed for the protein production during the end of the cultivation as the cells transitioned from stationary phase to death phase.

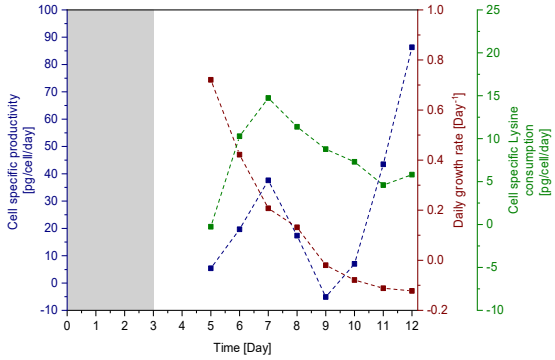
The negative impact of leucine on cells was not observed and this could be attributed to the low concentration of leucine available in the culture medium. Leucine consumption was similar to the one observed in case of isoleucine. The maximum metabolism of leucine was observed during transition of cells to stationary phase and death phase. Also, throughout the duration of the cultivation, leucine was constantly consumed indicating the use of the amino acid for daily functionality by the cells.

Lysine

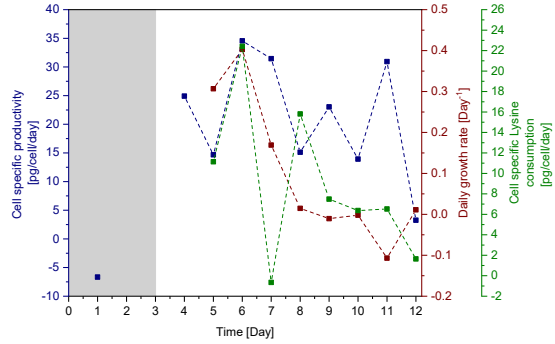
Concentration in different scale



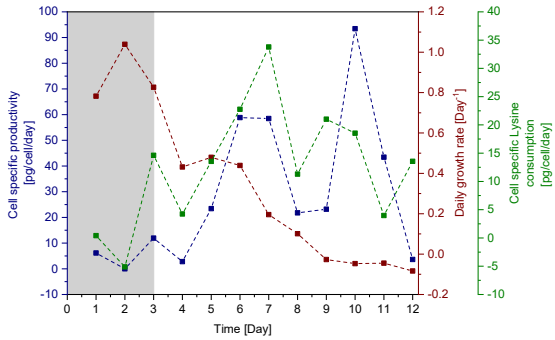
15 mL Microbioreactor (AMBR 15)



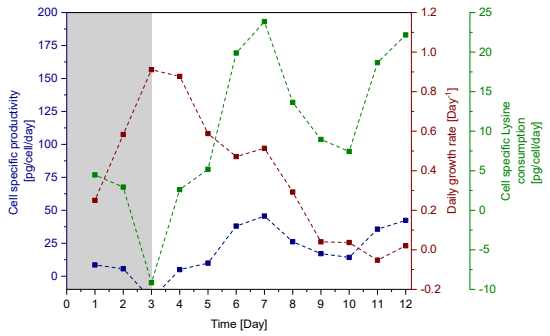
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

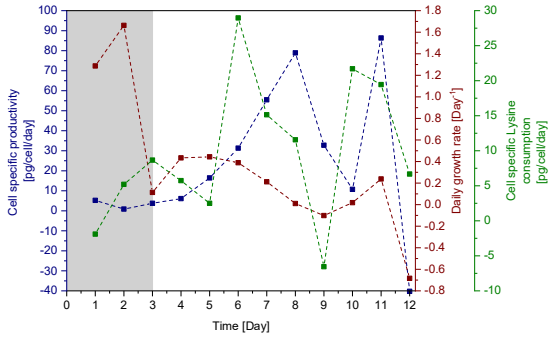


Figure 56 : Study of Lysine in different scales of bioreactor

One of the most commonly detected product during mAb production is C-terminal lysine. It has been studied that modification of C-terminal lysine has no substantial influence on

antibody structure, stability and function [105], however, the characteristic of lysine is important to ensure consistency of product quality. The study of the characteristic is also critical for monoclonal antibody process development and manufacturing [106]. *Zhang et. al.* demonstrated that mAb C-terminal lysine variant level increased with higher arginine and lysine concentration in culture medium.

Carrillo-Cocom et. al. [103] and *Gonzalez-Leal et. al*.[26] found that arginine and lysine have been well characterized in cell growth and monoclonal antibody production. They also stabilized mAb during purification and formulation process [107].

During the cultivation the initial concentration of lysine in the production medium ranged from 250 to 400 mg/L. The amount of lysine in the feed medium was measured to be 3645.35 mg/L. A fraction of this amount was added to the different bioreactors depending on the scale of the reactor. The amino acid was consumed during the batch phase and it was accumulated during fed batch until day 5. The cells consumed lysine while transitioning from exponential phase to stationary phase and was constant during the end of the stationary phase. The culture was supplemented with required amino acid and it was consumed by the cells. The cell specific consumption rate for each scale was measured and explained in detail in the sub section.

- 15 mL Microbioreactor (AMBR 15)

The amount of lysine added during the fed batch to the 15 mL microbioreactor was 1.35 mg per day. The maximum consumption of lysine was observed on day 7 with a value of 14.74 pg/cell day. The consumption gradually declined; however, the cells constantly consumed the amino acid. In order to study the impact on cell growth in detail, data from the first 4 days would be required.

- 500 mL Shake Flask

Shake flask samples were taken day 4 onwards and the data for the batch phase was missing. The amount of lysine added during fed batch was measured to be 13.4 mg per day from day 3 to day 11. The peak for growth rate, cell specific productivity and cell specific lysine consumption was measured to be on day 6 with a value of 0.40 Day^{-1}, 34.55 pg/cell/day and 22.42 pg/cell/day respectively. The consumption of lysine gradually declined, but was constantly being consumed till the end of the cultivation.

- 2 L Glass Bioreactor

The amount of lysine added to the 2 L bioreactor was measured to be 185.74 mg per day. The consumption of lysine increased on 2 days, i.e., on day 3 (14.62 pg/cell/day) and day 7 (33.78 pg/cell/day). These days correspond to the shift of phase from lag to exponential phase and exponential phase to stationary phase. The cells constantly consumed lysine until the end of the cultivation; indicating the use of lysine in the cell growth and protein production.

- 10 L stainless steel bioreactor

The longer lag phase in the bioreactor also effected the consumption of lysine and this was observed in the graph depicting the three attributes. The consumption declined until the end of batch phase. The amount of lysine added to the 10L reactor during the fed batch was measured to be 928.68 mg per day. During fed batch, the consumption gradually increases until a peak on day 7 (23.86 pg/cell/day) was reached. The consumption rate remained above 5 pg/cell/day till the end of the cultivation, indicating the use of lysine till the end of the cultivation.

- 50 L Single-use Stirred tank reactor (STR)

The amount of lysine added to the 50 L bioreactor during the fed batch was 4643.06 mg per day until day 11. The consumption reached a peak value on day 6 with a value of 28.96 pg/cell/day. Lysine was available and consumed till the end of cultivation, indicating the use of lysine for cell growth and protein production.

From the current study, use of lysine for cell growth and protein production was evident. The cells constantly consumed lysine until the end of cultivation. A bolus of lysine can be provided to the culture to test the impact of excess of lysine in the medium and to test if the concentration in the feed medium can be further optimized.

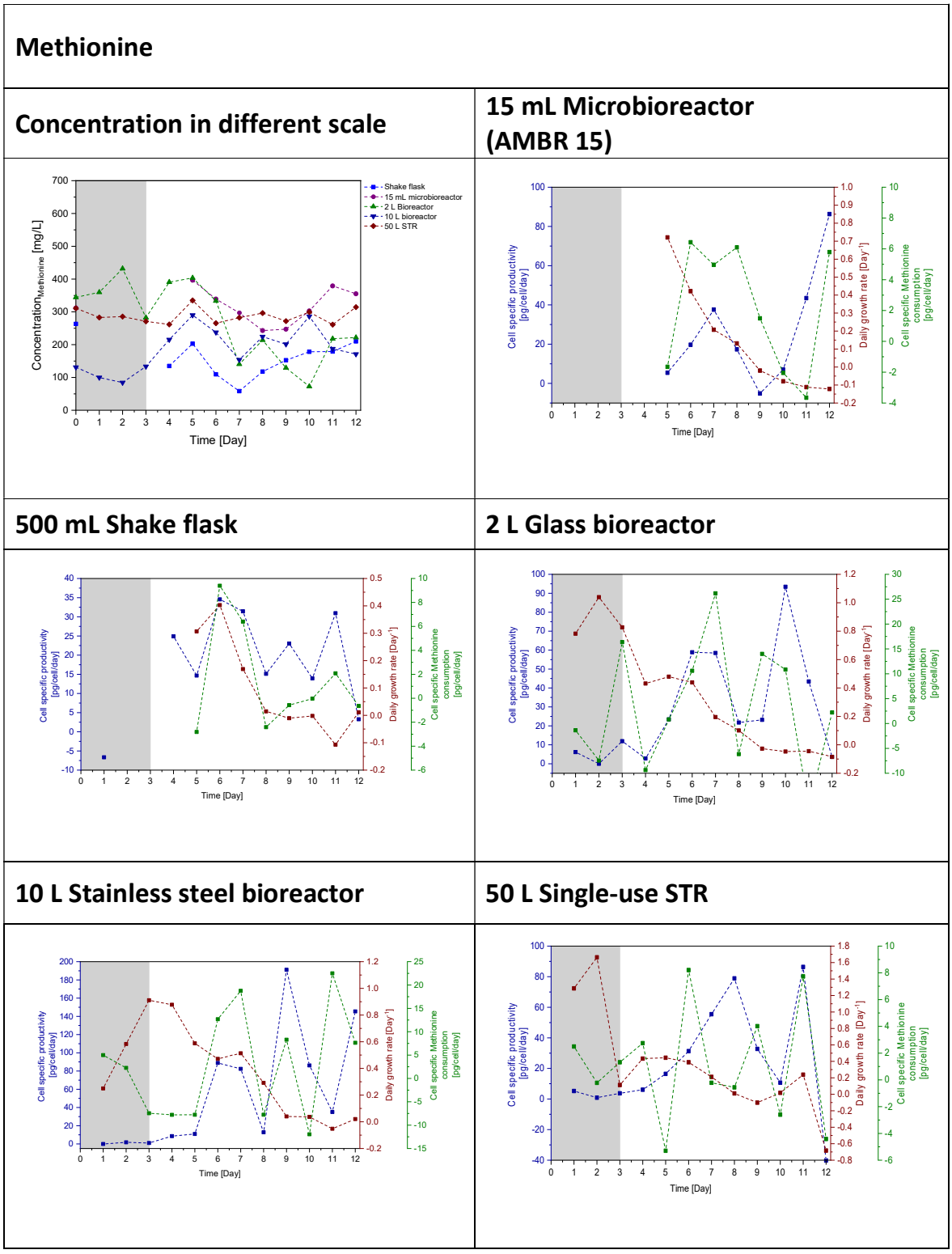


Figure 57 : Study of Methionine in different scales of bioreactor

Methionine is sulphur containing amino acid present in peptides and proteins. It is well known for its role in protein initiation. It is readily interchangeable with other residues such as leucine and valine [108]. It was established by *Kim et. al*. that methionine residues in

protein are positioned such that a hydrophobic bond is established between their disulfur atoms and rings of aromatic residues [109]. These along with cysteine influence the structural integrity and stability of proteins [108].

The amount of methionine in the production medium ranged from 150- 350 mg/L and 80 mg/L in feed medium. The percentage of methionine in the monoclonal antibody is less than 1 % the total amino acid in the sequence. The requirement of this amino acid was satisfied in the medium and this can be shown in Figure 57. As seen in figure, the concentration in the medium remained constant through the entire course of cultivation.

- 15 mL Microbioreactor (AMBR 15)

The amount of methionine added during fed batch to AMBR 15 was measured to be 0.33 mg per day. The data for batch phase was missing due to insufficient amount of sampling during initial stage of the cultivation. The consumption of methionine reached a peak on day 6 with a value of 6.43 pg/cell/day. The consumption gradually decreased day 9 onwards.

- 500 mL Shake Flask

Methionine was added to shake flask through the feed medium. The amount added during the fed batch was measured to be 3.31 mg per day. The peak consumption was observed on day 6 with a value of 9.40 pg/cell/day. The consumption gradually decreased and was negligible during the stationary phase. The metabolism increased on day 11 with a value of 2.1 pg/cell/day. This peak corresponded to the increase in cell specific productivity, however, the consumption was not significant.

- 2 L Glass Bioreactor

Methionine added to the 2 L bioreactor was measured to be 45.85 mg per day during the fed batch phase. The samples were measured from day 0 and the results were represented in the figure. The peak in consumption was observed on day 3 with an increases upto 16.4 pg/cell/day. The next peak was observed on day 7 with a value of 26.18 pg/cell/day. At the highest peak of cell productivity, the consumption of methionine was measured to be 10.85 pg/cell/day; indicating the use of methionine for protein production (in lower concentration). This corresponded to the requirement of methionine in the structure of the protein.

- 10 L stainless steel bioreactor

The amount of methionine added to the 10 L bioreactor was measured to be 229.21 mg per day. The peak of consumption was observed on day 7 with a value of 18.78 pg/cell/day. The maximum cell specific productivity was measured on day 9 and the amino acid consumption on the day was measured to be 8.30 pg/cell/day.

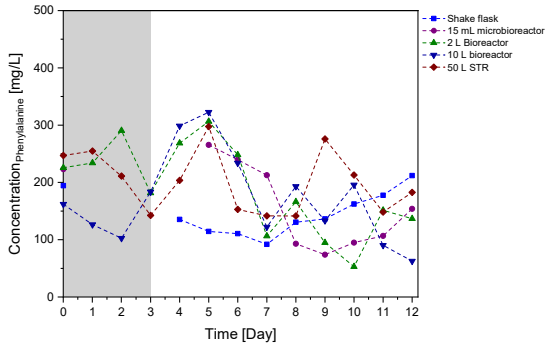
- 50 L Single-use Stirred tank reactor (STR)

Feed medium containing the essentials for cells was added to the bioreactor day 3 onwards until day 11. The amount of methionine added to the 50 L bioreactor was 1146 mg per day and consumption was measured based on the amount present in the medium during the entire course of cultivation. The peak consumption was observed on day 6 with a value of 8.2 pg/cell/day. The consumption gradually declined, however, the peak of cell specific productivity corresponded to the increase in methionine consumption on day 11.

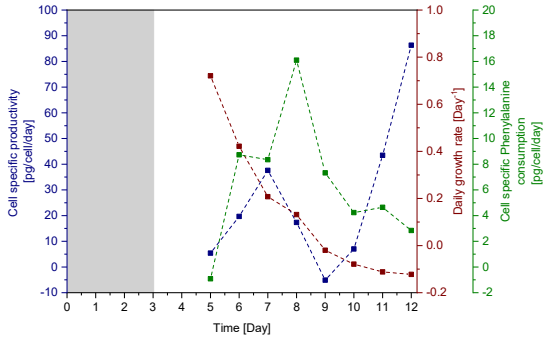
In all the different scale of bioreactors, a peak was observed when cells transition from exponential phase to stationary phase. An increase in consumption was also observed at the peak of cells specific productivity, indicating the use of certain concentration of methionine for the protein production. More tests would have to be performed to study the impact on protein stability.

Phenylalanine

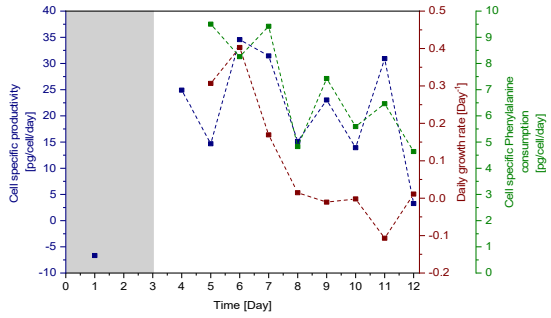
Concentration in different scale



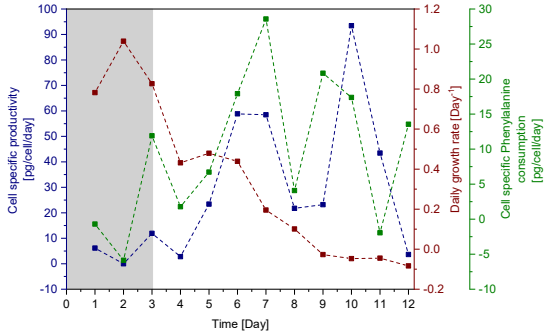
15 mL Microbioreactor (AMBR 15)



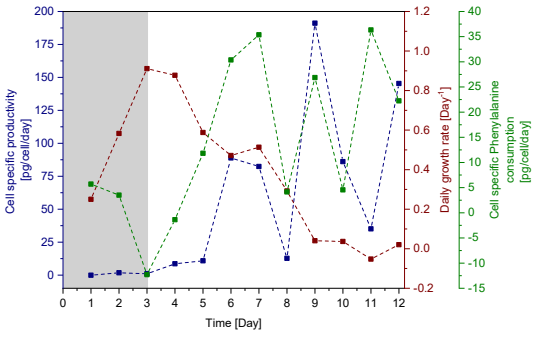
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

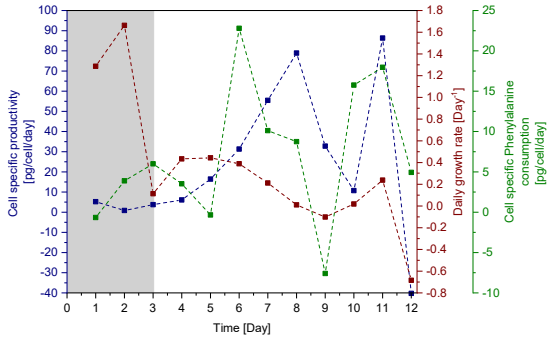


Figure 58 : Study of Phenylalanine in different scales of bioreactor

Phenylalanine if present in high concentration can have negative effect on mAb titre response and integral viable cell concentration response [87]. Low concentration also has a

negative effect on the cell growth. *Lazard et. al.* lowered the phenylalanine concentration to 2 µM from 200 µM and the cell growth was arrested [86]. The ideal concentration for phenylalanine is recommended to be maintained between 0.5 – 1 mM in fed batch to ensure optimum cell growth and mAb titre response [15]. Another property of phenylalanine is that it can be substituted as tyrosine but is less effective [110]. With the knowledge of these properties the impact of phenylalanine was studied on the CHO DG44 cell line used for the project.

The Initial concentration of phenylalanine in the production medium was measured to range between 150 to 250 mg/L and the amount in feed medium was 3105 mg/L. A fraction of the amount was added to the different scale of bioreactors.

The amino acid was gradually consumed until end of batch phase and was accumulated in the medium till day 5. The concentration of phenylalanine was maintained between the initial concentration till the end of the cultivation.

- 15 mL Microbioreactor (AMBR 15)

Phenylalanine in the medium fed to the bioreactor per day from day 3 to day 11 was measured to be 1.062 mg/day. The peak consumption occurred on day 8 with a value of 16 pg/cell/day followed by a steep decrease in the consumption. Phenylalanine was constantly metabolised until the end of the cultivation, indicating the use of amino acid for daily functionality and protein production during the stationary phase.

- 500 mL Shake Flask

The amount of phenylalanine added to 150mL shake flask during the fed batch was measured to be 10.56 mg per day until day 11. The peak of phenylalanine consumption was observed on day 7, 9 and 11 with a value of 9.42 pg/cell/day, 7.43 pg/cell/day and 6.46 pg/cell/day respectively. The amino acid was metabolised until the end of cultivation, indicating the use of phenylalanine in protein production and integral viable concentration.

- 2 L Glass Bioreactor

Phenylalanine added to the 2 L bioreactor per day was measured to be 146.62 mg per day. The consumption reached a maximum peak on day 7 with a value of 29 pg/cell/day. The

amino acid was consumed till the end of the cultivation. The cell specific productivity reached a peak on day 9 with a value of 93.44 pg/cell/day and the corresponding phenylalanine consumption was measured to be 17.37 pg/cell/day.

- 10 L stainless steel bioreactor

The amount of phenylalanine added to the 10 L bioreactor is measured to be 731.62 mg per day during the fed batch phase. The consumption gradually increased till day 7 with a value of 35.36 pg/cell/day. The consumption of phenylalanine increased on day 9 with an increase in the cell specific productivity.

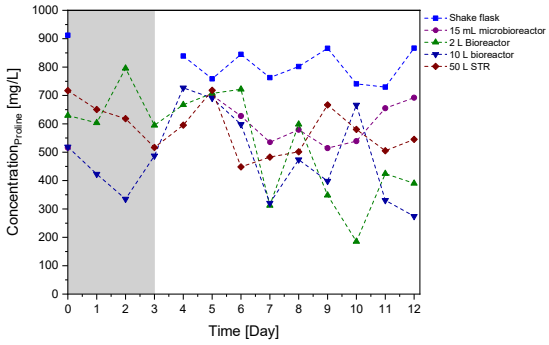
- 50 L Single-use Stirred tank reactor (STR)

The feed medium contained phenylalanine and the amount added to the 50 L bioreactor was measured to be 3658 mg per day. The metabolism was negligible until day 5 and the peak consumption of phenylalanine occurred on day 6 with a value of 22.80 pg/cell/day. The consumption of the amino acid increased at the end of cultivation too.

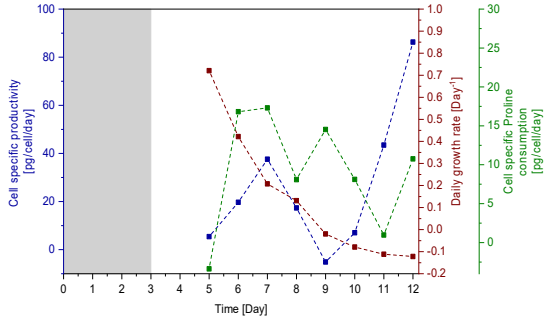
The percentage of phenylalanine present in the monoclonal antibody is 3 % the total amino acid. The consumption of the amino acid in the different scales of bioreactor demonstrated the use of it during the transition on day 7 and an increase in the peak again at the end of the cultivation. It can be suggested that phenylalanine is extensively used by the cells for maintaining IVCC and production of protein during the cultivation.

Proline

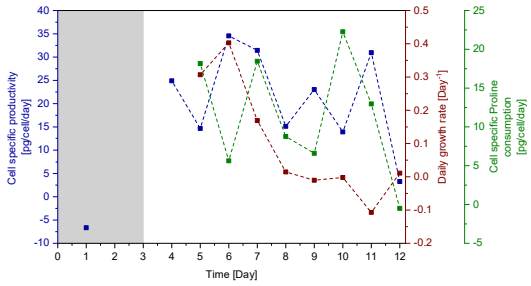
Concentration in different scale



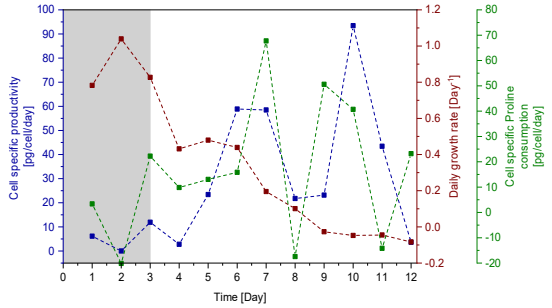
15 mL Microbioreactor (AMBR 15)



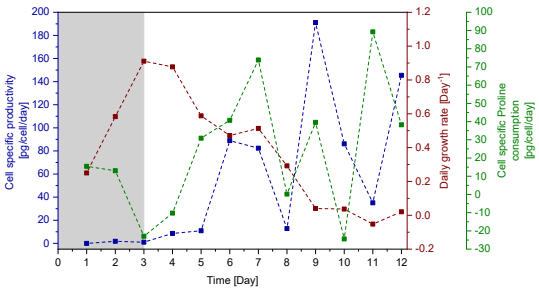
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

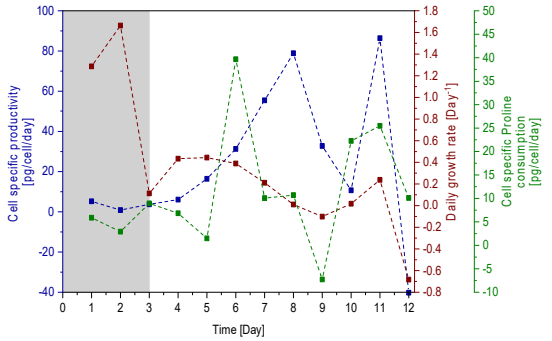


Figure 59 : Study of Proline in different scales of bioreactor

Addition of proline is known to positively impact metabolic parameters including glutamine utilization, final ammonium levels, glucose consumption and lactate production.

Additionally, proline can also be used to mitigate toxic effects on cell growth and protein productivity and quality [13]. Chen and Harcum [13] tested the effect of proline on CHO cell growth and protein quality. They found the culture supplemented with proline had lower growth rates and final cell density than control but significantly improved the final cell density when compared to the negative control. Specifically the addition of proline improved the final cell density by 20 % when compared to the negative control. Proline can be converted to glutamate by ATP independent glutamate semi aldehyde dehydrogenase. The addition of proline helped cells produce α-ketoglutarate via glutamate, during which only single ammonia molecule per proline was generate. In contrast, glutamine consumption generated two molecules per glutamine molecule. Thus substituting proline instead of glutamine could help cells have an active TCA cycle with reduced ammonium production [13].

The initial amount of proline in production medium ranged from 500 to 700 mg/L and feed medium contained 4760 mg/L of proline. The concentration of proline in the supernatant remained constant and was metabolised as much as required by the cells for the mAb production since there was no change in metabolism due to deficiency of any other amino acid. This was discussed in detail in the sub section with respect to the different scale of bioreactors.

- 15 mL Microbioreactor (AMBR 15)

Proline was added to AMBR 15 via the feed medium and the amount added during feed was measured to be 1.77 mg per day. The maximum consumption of proline was observed on day 7 with a value of 17.3 pg/cell/day and the cell specific productivity on day 7 was measured to be 37.6 pg/cell/day. Proline was consumed throughout the cultivation and was used in cell growth and protein production.

- 500 mL Shake Flask

The amount of proline added per day was measured to be 17.62 mg per day during the fed batch phase of the cultivation. The cells consumed 18.45 pg/cell/day of proline on day 7 and a peak in consumption was observed on day 10 with the value increasing to 22.26 pg/cell/day. The correlation between the three parameters was not distinctly established and this could be due unavailable samples during the cultivation.

- 2 L Glass Bioreactor

In the 2 L bioreactor, sample was taken every day and the results were compared in the figure. The amount of proline added to the bioreactor was measured to be 244.25 mg per day from day 3 till day 11. The amino acid consumption increased during the end of batch phase with a measurement of 22.2 pg/cell/day and the peak consumption was observed on day 7 (67.71 pg/cell/day). The consumption of proline at the peak of cell specific productivity was measured to be 40.69 pg/cell/day.

- 10 L stainless steel bioreactor

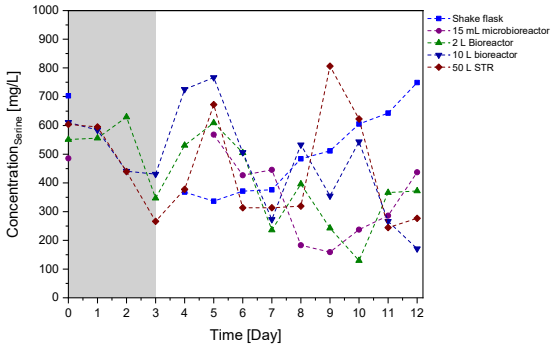
The quantity of proline added to the 10 L bioreactor was 1221.26 mg per day and this was supplemented to the bioreactor through FMA and FMB. The consumption of proline increased on day 7, day 9 and day 11 with 73.8 pg/cell/day, 39.6 pg/cell/day and 89.24 pg/cell/day, respectively. The protein production per unit of cell was maximum on day 9 and proline was consumed upto 39.6 pg/cell/day indicating the use of the amino acid in protein production. The increase of consumption on day 11 may be used for stability of the protein structure; however, this has to be further investigated.

- 50 L Single-use Stirred tank reactor (STR)

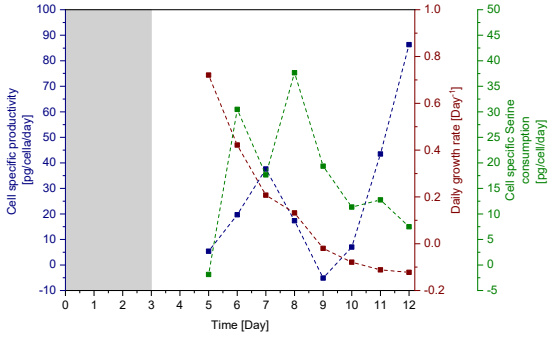
Approximately 6106 mg of proline was added per day during the fed batch of the cultivation. The amino acid was constantly metabolised and the concentration remaining in the supernatant was measured using RP-HPLC. The peak consumption was observed on day 6 with a value of 39.66 pg/cell/day. The consumption of proline on day 11 increased and this corresponded to the increase in cell specific productivity; suggesting the use of proline for protein production and stability.

Serine

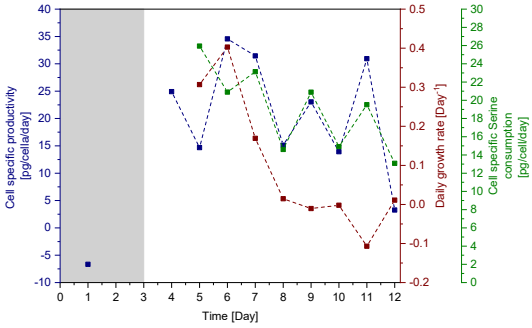
Concentration in different scale



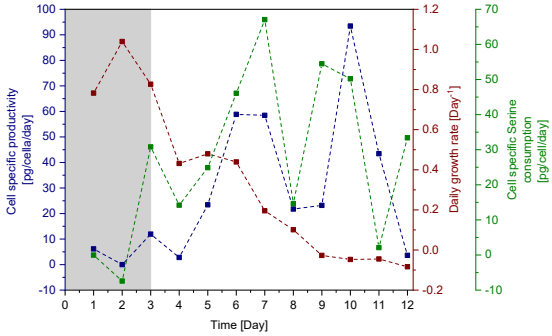
15 mL Microbioreactor (AMBR 15)



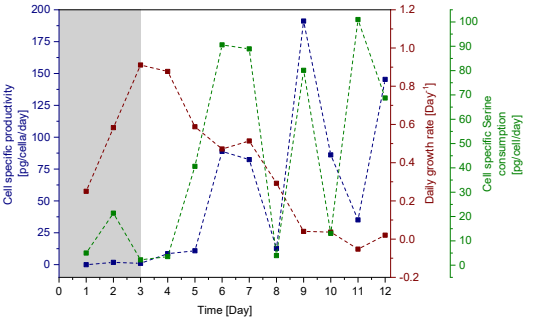
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

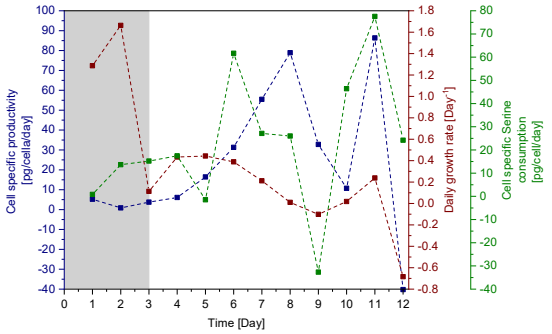


Figure 60 : Study of Serine in different scales of bioreactor

Serine provides the essential precursors for the synthesis of proteins, lipids and nucleic acids. High concentration of serine has the highest negative impact on final mAb titre

response [87]. It is also the amino acid present in the highest percentage in the monoclonal antibody comprising of 13% the total amino acids.

The initial amount of serine in the production medium ranged from 500 to 700mg/L and 7850 mg/L in feed medium. The amino acid was gradually consumed during the lag phase and accumulated in the medium during the exponential phase. It was constantly supplied to the bioreactors and consumed constantly by the cells. The concentration did not deplete below 100 mg/L, indicating sufficient supply of serine for the normal functionality of the cells. The cell specific consumption was compared to the growth rate and cell specific productivity to establish a correlation between the three parameters and understand the influence of serine in detail.

- 15 mL Microbioreactor (AMBR 15)

Addition of serine via feed medium was estimated to be about 3.14 mg added per day during the fed batch phase of the cultivation. A peak was observed on day 8 with a consumption rate of 37.65 pg/cell/day. The consumption of serine remained above 5 pg/cell/day till the end of the cultivation, suggesting the use of serine for protein production in the cultivation.

- 500 mL Shake Flask

The quantity of serine added to 150 ml shake flask was measured to be 31.29 pg/cell/day. The data for the batch phase was missing, however, it was noted that the consumption of serine throughout the cultivation was above 12 pg/cell/day. A peak was observed on days 7, 9 and 11 with a value of 23.13 pg/cell/day, 20.89 pg/cell/day and 30.94 pg/cell/day. These peaks corresponded to the peak in cell specific protein production indicating the use of serine in larger amount for protein production.

- 2 L Glass Bioreactor

The amount of serine added to the 2 L bioreactor was measured to be 433.70 mg per day during the fed batch phase. A peak was observed on day 3 when the conditions in the bioreactor switched from batch phase to fed batch phase. A maximum peak in consumption was measured on day 7 with a value of 67 pg/cell/day. On day 10, the cell specific productivity was at a peak and the serine consumption on that day was measured to be

50.20 pg/cell/day.

- 10 L stainless steel bioreactor

Serine added to the bioreactor during fed batch was measured to be 2168.49 mg per day. The consumption of serine increased on day 6, 9 and 11 with values 90.55 pg/cell/day, 80.12 pg/cell/day and 101 pg/cell/day. The cell specific productivity reached a peak on day 9 with cell consumption of serine reaching a rate of 80.12 pg/cell/day.

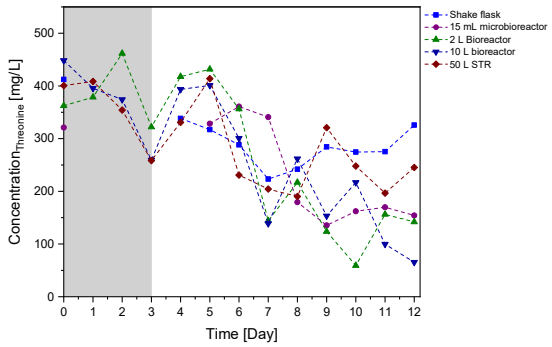
- 50 L Single-use Stirred tank reactor (STR)

The amount of serine added to the 50 L bioreactor was 10842 mg per day during the fed batch phase until day 11. The serine consumption rate was measured and the peak was observed on day 6 (61.68 pg/cell/day) followed by a sharp increase in consumption on day 11(77.54 pg/cell/day). The day of highest cell specific productivity saw an increase in the consumption of serine too.

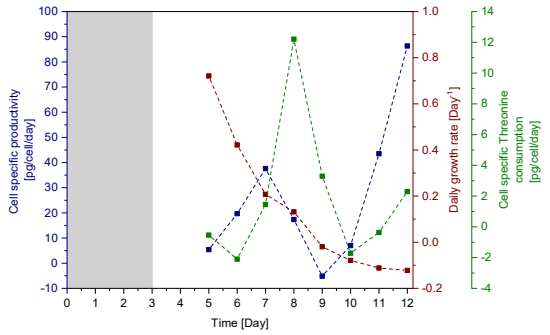
Serine constitutes to 12.78 % of the total amino acid present in the monoclonal antibody of interest. The different scales of bioreactors saw a typical trend of constantly metabolising serine throughout the cultivation and the peak consumption observed on days of transition into the stationary phase and also during the high cell specific productivity. The concentration of serine in the medium was sufficient for carrying forward basic functionality of the cells along with protein production, however, from this study the strong influence of serine on the protein production can be concluded.

Threonine

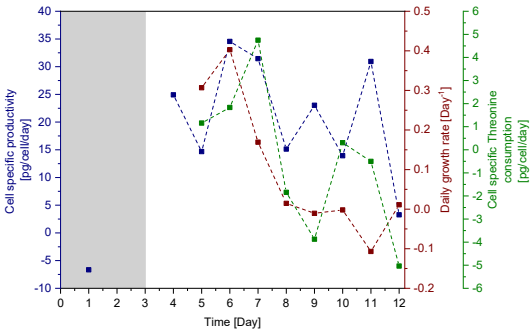
Concentration in different scale



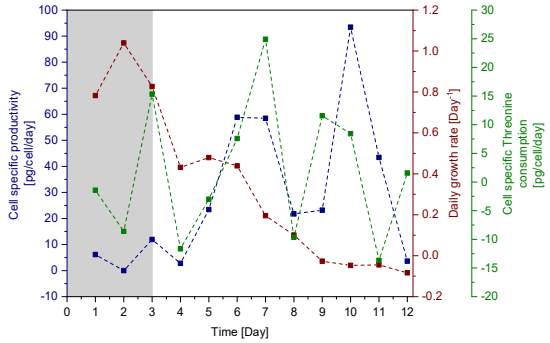
15 mL Microbioreactor (AMBR 15)



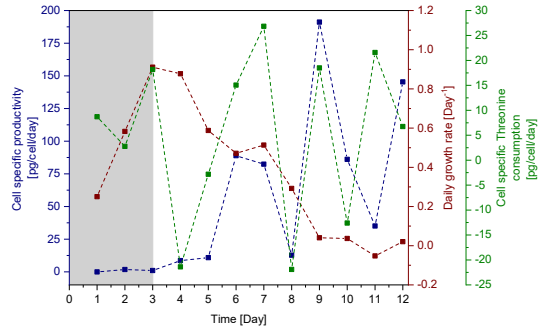
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

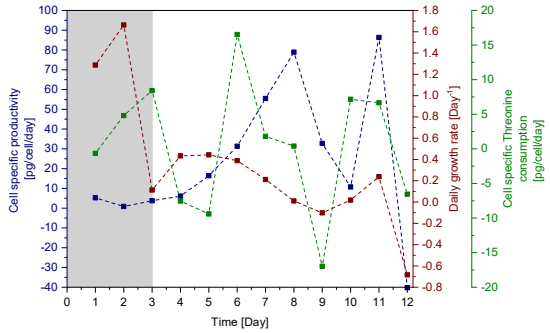


Figure 61: Study of Threonine in different scales of bioreactor

Addition of threonine is known to positively impact metabolic parameters including

glutamine utilization, final ammonium levels, glucose consumption and lactate production. Additionally, threonine can also be used to mitigate toxic effects on cell growth and protein productivity and quality [13]. Chen and Harcum tested the effect of threonine on CHO cell growth and protein quality. They found the culture supplemented with threonine had lower growth rates and final cell density than control, but, significantly improved the output parameters than the negative control. Specifically the addition of threonine improved the final cell density by 15 % when compared to the negative control. Glycine and threonine metabolism are connected where glycine can be converted to threonine. Glycine and threonine degradation may result in an increased pyruvate level and thereby increase the flux through TCA cycle in turn improving the cell energetic. [13]

The amount of threonine in the production medium ranged from 300 to 450 mg/L and 160 mg/L was supplemented using feed medium. The value varied depending upon the volume of the bioreactors. The concentration of the amino acid decreased to approximately 250 to 300 mg/L at the end of day 3. The amount accumulated until day 5 and gradually decreased until the end of the cultivation. The amount, however, was not limited and was therefore sufficiently present in the culture for the cells for cell growth and protein production.

- 15 mL Microbioreactor (AMBR 15)

The concentration of threonine was constantly depleted in the medium, however, the amount added to the bioreactor was measured to be 0.0227 mg per day during the feeding phase of the cultivation. The consumption reached a peak on day 8 with a value of 12.2 pg/cell/day. The consumption was negligible at the end of the cultivation

- 500 mL Shake Flask

Threonine is one of the major amino acid present in the monoclonal antibody; however, the addition of this amino acid was as low as 0.224 mg per day during the fed batch phase. A peak in the consumption was observed on day 7 with a value of 4.74 pg/cell/day. The consumption of threonine was negligible during the stationary phase. It can be speculated that threonine was used for the initial cell growth and initiating the protein production.

- 2 L Glass Bioreactor

The amount of threonine added to the 2 L bioreactor was measured to be 3.114 mg per day from day 3 to day 11. The consumption peaked on day 3 (15.33 pg/cell/day) and day 7 (24.9 pg/cell/day), the days when the cells transition from lag phase to exponential phase and exponential phase to stationary phase, respectively. During the peak cell specific productivity, the threonine consumption was recorded to be 8.44 pg/cell/day.

- 10 L stainless steel bioreactor

15.57 mg of threonine was added per day to the 10 L bioreactor during fed batch phase. A peak was observed on day 3 (18.17 pg/cell/day), day 7 (24.9 pg/cell/day), day 9 (11.57 pg/cell/day) and day 11 (21.58 pg/cell/day). On the peak of cell specific protein production, the threonine consumption was recorded at 11.57 pg/cell/day.

- 50 L Single-use Stirred tank reactor (STR)

The amount of threonine added to the 50 L bioreactor was measured at 77.44 mg per day from day 3 until day 11. The consumption peaks on day 7 (16.5 pg/cell/day) and the rate decreases over the remaining time of cultivation. The threonine consumption rate on the day of peak of specific protein production was recorded at 6.65 pg/cell/day.

The consumption of threonine may not be as high as the other amino acids but the consumption was comparatively significant on the day of peak cell specific productivity; indicating the use of threonine for initiating protein formation and the protein production. The concentration of threonine was sufficiently present in the medium for the culture and would not require any further optimization, though the relevance on the protein production and metabolism throughout the cultivations is significant .

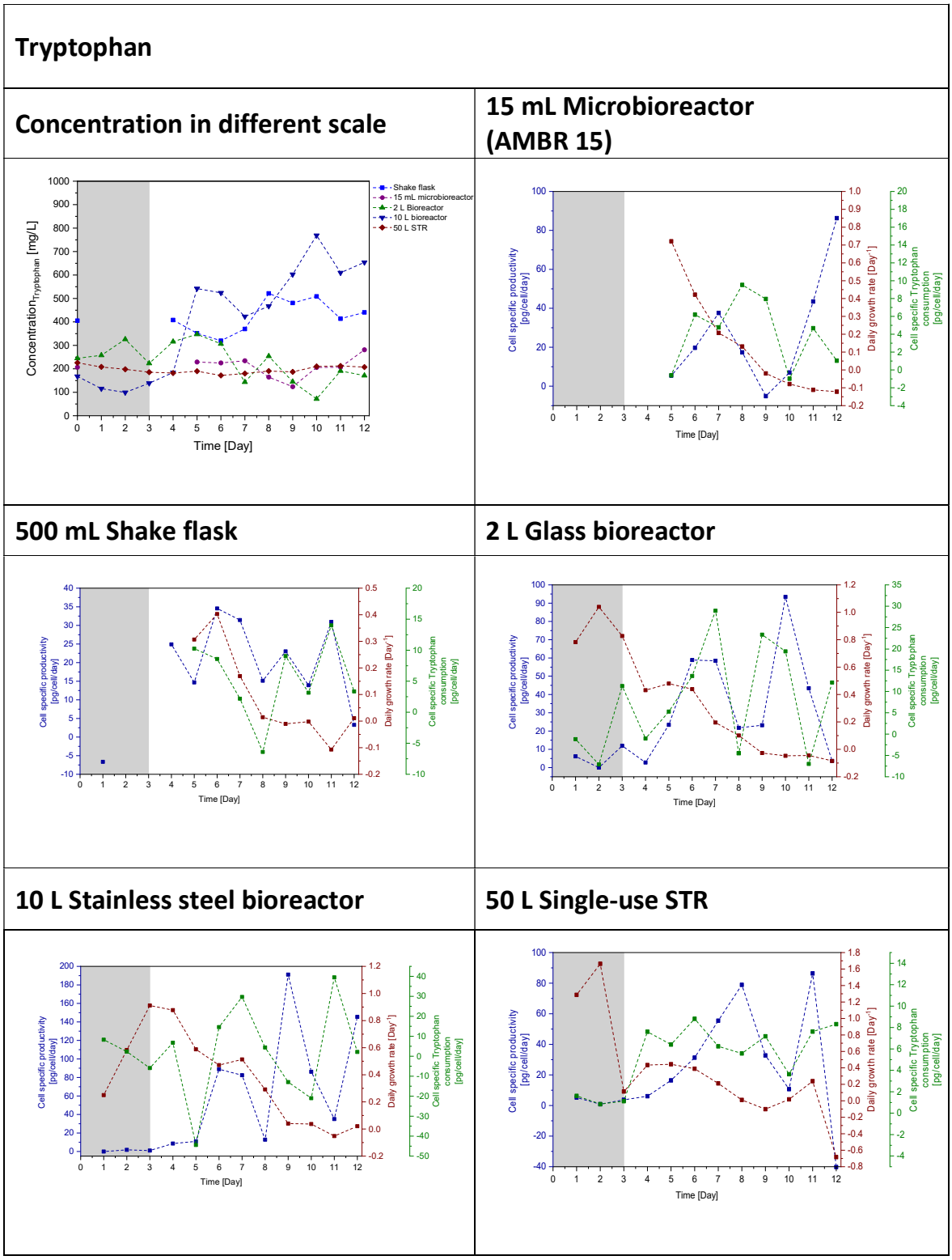


Figure 62 : Study of Tryptophan in different scales of bioreactor

Tryptophan is a precursor to melatonin, vitamin B3 and neurotransmitter serotonin [111]. The impact of tryptophan on the mammalian cells (predominantly mouse LM cells) was studied by Michael Brunner [112]. Brunner reported that limitation of tryptophan in growth

medium resulted in arrest in cell growth, presumably in G1 phase. This was reversible and the growth of cells restored on supplementing with tryptophan. Suggesting the use of tryptophan predominantly for cell growth and proliferation.

The percentage of tryptophan in the monoclonal antibody was calculated to be less than 2 % the total amino acids present in the protein of interest.

The amount of tryptophan present in the production medium ranged from 150 to 400 mg/L. Feed medium B contained 17681 mg/L and feed medium A contained 414 mg/L. However, the total amount of tryptophan added was only a fraction of it because only 10 % of feed medium B was added when compared to feed medium A. The concentration of amino acid remained constant throughout the cultivation.

- 15 mL Microbioreactor (AMBR 15)

The amount of tryptophan added to the AMBR 15 was measured to be 0.91 mg per day. The consumption of tryptophan reached a peak on day 8 with a value of 9.52 pg/cell/day. The cells constantly metabolised the tryptophan present in the medium till the end of the cultivation.

- 500 mL Shake Flask

Tryptophan added to the cells in the bioreactor was measured to be 8.73 mg per day during the fed batch phase. The impact of tryptophan on the cells during the batch phase was unavailable due to insufficient sample. The peak consumption of tryptophan was measured to day 9 (9.05 pg/cell/day) and day 11 (14.01 pg/cell/day); these days correspond to the increase in cell specific productivity.

- 2 L Glass Bioreactor

During the fed batch, 121.98 mg of tryptophan was added to the 2 L bioreactor from day 3 to day 11. A peak in consumption was observed on day 3 (11.27 pg/cell/day), day 7 (28.93 pg/cell/day) and day 9 (23.32 pg/cell/day). The consumption rate of tryptophan on the day of peak cell specific productivity was measured to be 19.42 pg/cell/day.

- 10 L stainless steel bioreactor

The amount of tryptophan added to the 10 L bioreactor was measured to be 610 mg per day during the fed batch phase. The amino acid was consumed and reached a peak on day 7 (29.67 pg/cell/day) and day11 (39.47 pg/cell/day). However, no direct correlation between the three attributes was observed.

- 50 L Single-use Stirred tank reactor (STR)

3032 mg of tryptophan was added to 50 L bioreactor during the fed batch phase of the cultivation. The amino acid was constantly consumed throughout the cultivation and no significant peak was observed during the cultivation. The consumption was above 4 pg/cell/day until the end of the cultivation.

As per studies conducted by Brunner, tryptophan was predominantly used of the cell growth and proliferation. Though, a direct correlation between the growth rate, protein production and tryptophan consumption was not established, it was evident that the amino acid was constantly metabolised by the cells for daily functionality and growth.
Throughout the cultivation, sufficient amount of tryptophan was available for culture suggesting the focus for medium optimization can be switched to other amino acids.

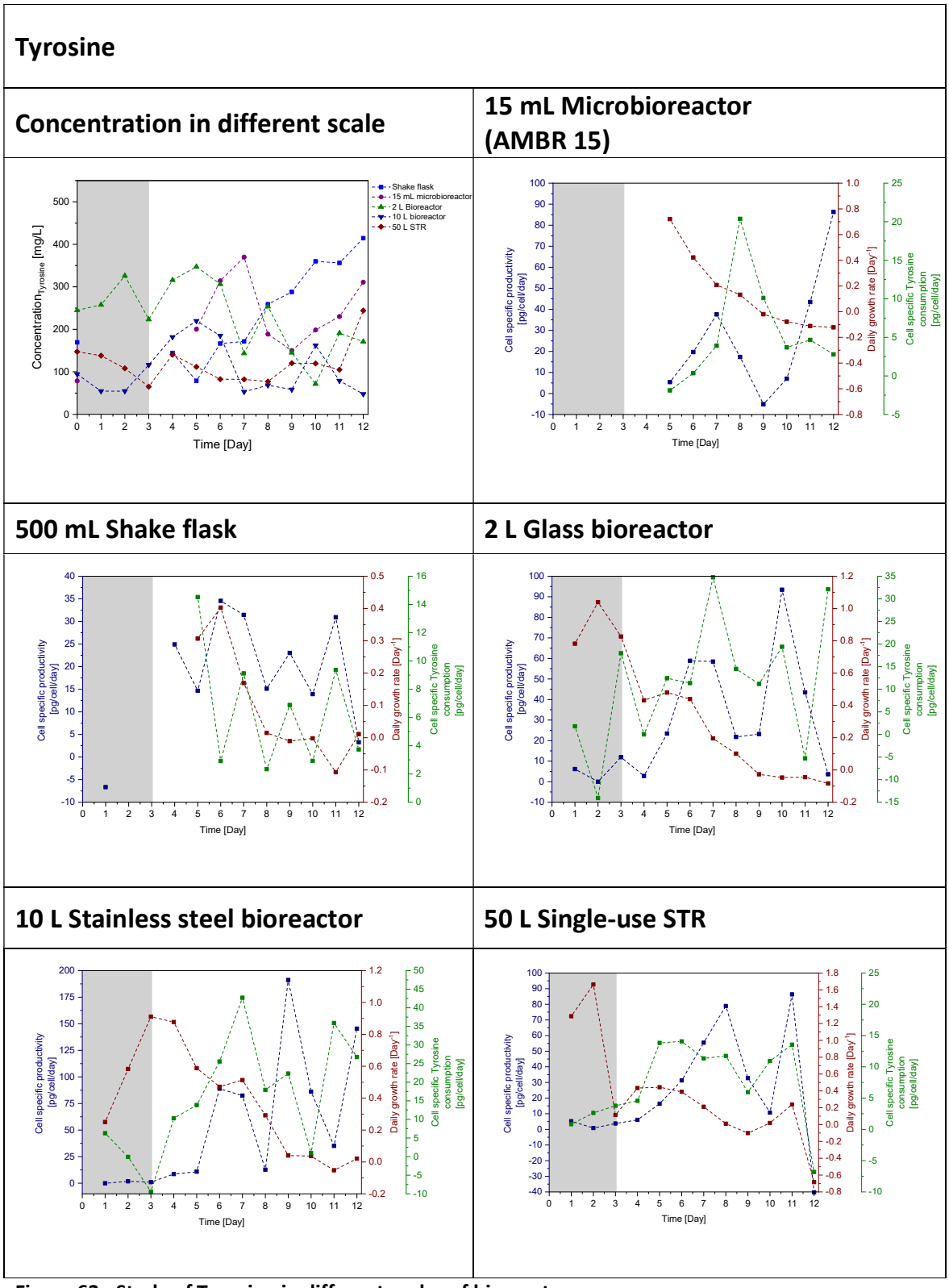


Figure 63 : Study of Tyrosine in different scales of bioreactor

Addition of tyrosine in the medium has a positive impact on the final mAb titre response and integral viable cell concentration response [87]. The influence of tyrosine is studied in this

segment.

The amount of tyrosine in the production medium ranged from 100 to 250 mg/L. The amount of tyrosine present in feed medium A was 262 mg/L and feed medium B contained 29.155 mg/L, however, the bioreactor was supplemented with FMA and FMB which constituted for about 10% of FMA and therefore the concentration of tyrosine added to the culture was negligible in comparison.

Figure 63 showcased the concentration of tyrosine in the medium during the entire course of the cultivation. The concentration throughout the run did not drop below 50 mg/L and it was sufficiently available in the medium for the cells to use it for cell growth and protein production.

- 15 mL Microbioreactor (AMBR 15)

The quantity of tyrosine added to the AMBR 15 bioreactor was measured to be 1.33 mg per day during the fed batch phase of the cultivation. The consumption of tyrosine gradually increased during the fed batch and peaked on day 8 with a value of 20.37 pg/cell/day. The amino acid was consumed throughout the cultivation.

- 500 mL Shake Flask

Tyrosine added to the 150 mL shake flask mounted to 12.7 mg per day from day 3 to day 11. The amino acid was consumed above 2 pg/cell/day until the end of cultivation. A peak was observed on day 5 with a value of 14.53pg/cell/day. Towards the end of cultivation, the peak in cell specific productivity occurred on day 11 and the corresponding tyrosine consumption rate was recorded at 9.36 pg/cell/day.

- 2 L Glass Bioreactor

The amount of tyrosine added to the 2 L bioreactor was measured to be 177.83 mg per day during the fed batch phase. The consumption of tyrosine increased on day 3 (17.96 pg/cell/day) and day 7 (34.76 pg/cell/day). On day 10, the cell specific productivity reached a peak and the tyrosine consumption on day 10 was measured to be 19.39 pg/cell/day.

- 10 L stainless steel bioreactor

Tyrosine added to the 10 L bioreactor was measured to be 890 mg per day during the fed batch phase of the cultivation. The peak consumption rate was observed on day 7 with a value of 42.7 pg/cell/day. The amino acid consumption rate on the day of highest productivity of protein was measured to be 22.32 pg/cell/day.

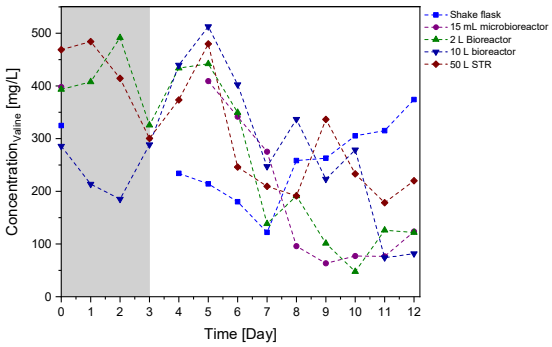
- 50 L Single-use Stirred tank reactor (STR)

The amount of tyrosine added to the 50 L bioreactor was measured to be 4416.65 mg per day from day 3 to day 11. The amino acid was constant consumed throughout the cultivation. There was a sharp decrease in consumption on day 12 and this was caused due to a drastic dip in the viable cells in the bioreactors. The tyrosine consumption on the day of peak of cell specific productivity was measured to be 13.54 pg/cell/day.

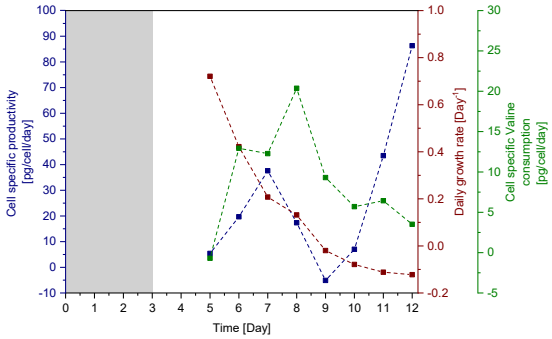
Tyrosine makes up 4.5 % of the total amino acid composition in the desired monoclonal antibody. The consumption in the different scale of bioreactor indicated a constant use of the amino acid to carry forward the basic functionality of the cell and to enhance the protein production. Sufficient amount of tyrosine was present in the bioreactors, suggesting concentrating on other amino acids for the optimization of the feed medium to enhance the cell growth and protein production.

Valine

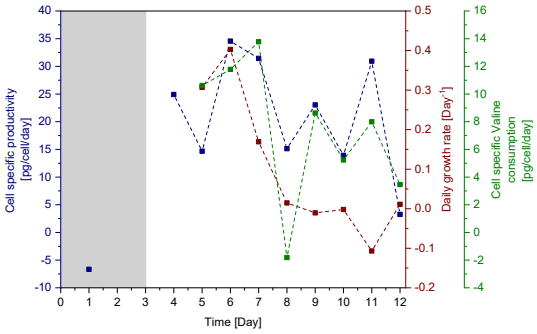
Concentration in different scale



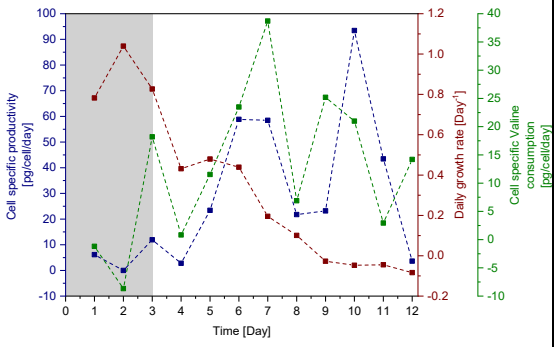
15 mL Microbioreactor (AMBR 15)



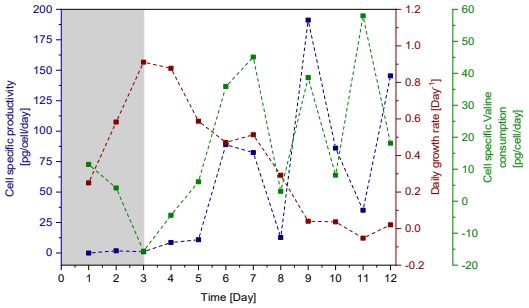
500 mL Shake flask



2 L Glass bioreactor



10 L Stainless steel bioreactor



50 L Single-use STR

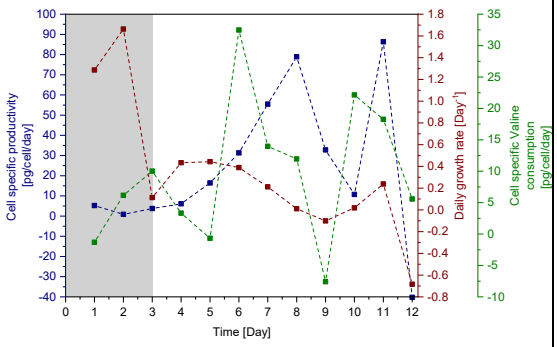


Figure 64 : Study of Valine in different scales of bioreactor

Valine is the second most abundant amino acid in the monoclonal antibody and it comprises of approximately 9.02 % of the total amount in the protein. However, high concentration of

valine in the culture medium could have a negative impact on the integral viable cell concentration response and final mAb titre response [87], also, deprivation of valine can result in an abrupt decreases in both RNA and protein synthesis [113]. The concentration of valine must be maintained at a range such that there is no adverse influence on the cell growth or the productivity due to the amino acid.

The initial concentration in production medium ranged from 300 to 450 mg/L and the amount in feed medium was 3100 mg/L. The amino acid was metabolised and reached a value of 300 mg/L, the concentration increased during fed batch and was consumed day 5 onwards. It was maintained at constant level day 7 onwards. Indicating the consumption was highest when the cells were transitioning from exponential phase to stationary phase. The results from the different scales were compared and the impact on cell growth and cell specific productivity was compared in subsection.

- 15 mL Microbioreactor (AMBR 15)

The amount of valine added to the 15 mL bioreactor was 1.24 mg per day during the fed batch phase of the bioreactor. Valine was constantly metabolised during the entire course of the cultivation. A peak in consumption rate was observed on day 8 with a value of 20.37 pg/cell/day. Towards the end of the cultivation, the consumption rate was nearly constant (on an average) at 5 pg/cell/day.

- 500 mL Shake Flask

Valine added to the shake flask was measured using the RP-HPLC and the amount of amino acid added to the system was 12.32 mg per day during the fed batch phase. A peak in valine consumption was observed on day 7 with a value of 13.78 pg/cell/day. The peak of cell specific productivity was noted on day 11 and the valine consumption rate on the day was measured to be 7.99 pg/cell/day.

- 2 L Glass Bioreactor

The amount of valine added to the 2 L bioreactor was measured to be 170.83 mg per day from day 3 to day 11. . The consumption of valine increased on day 3 (18.20 pg/cell/day) and day 7 (38.7 pg/cell/day). On day 10, the cell specific productivity reached a peak and the

amino acid consumption on day 10 was measured to be 20.98 pg/cell/day; suggesting the use of valine in protein production.

- 10 L stainless steel bioreactor

854.14 mg of valine was added daily during the fed batch phase of the cultivation. A peak in the consumption was observed on day 7 (45.06 pg/cell/day), day 9 (38.67 pg/cell/day) and day 11 (57.95 pg/cell/day). The amino acid consumption rate on the day of highest productivity of protein (day 9) was measured to be 38.67 pg/cell/day.

- 50 L Single-use Stirred tank reactor (STR)

The amount of valine added to the 50 L bioreactor was measured to be 4270.7 mg per day from day 3 to day 11. The amino acid was constant consumed throughout the cultivation. A peak in the consumption was noted on day 6 with a value of 32.48 pg/cell/day. The valine consumption on the day of peak of cell specific productivity was measured to be 18.27 pg/cell/day.

The monoclonal antibody is composed of 9 % valine and from the data presented in this section; it was observed that the amino acid was constantly being metabolised for the protein production. However, sufficient amount of valine is present in the medium thereby reducing the chance to optimize the concentration further.

6 List of Figures

7 List of Tables

8 References

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9 List of Publications

M. Allers, T. Reinecke, **T. Nagraik**, D. Solle, K. Bakes, M. Bergers, T. Scheper, S. Zimmermann, Differential Inductive Sensor for Continuous Non-Invasive Cell Growth Monitoring in Disposable Bioreactors, Multidisciplinary Digital Publishing Institute, Proceedings, 2017.
DOI: 10.3390/proceedings1040518

T. Nagraik, A. Salcedo, D. Solle, T. Scheper, Process Optimization using High Throughput Automated Micro-Bioreactors in Chinese Hamster Ovary Cell Cultivation, Journal of Visualized Experiments, May 2020
DOI: 10.3791/60577

Poster

J. Schellenberg, **T. Nagraik**, J. Bahnemann, D. Solle, T. Scheper, Impact of stress on the productivity in mammalian cells, 4th Conference of Scientific Cooperation between Lower Saxony and Israel, Hannover, Germany

10 Resume

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11 Acknowledgment

The completion of the work was possible due to the support and guidance of innumerous people.

I would like to first thank Prof. Dr. Thomas Scheper for giving me the opportunity to conduct the doctoral studies at Institute of Technical Chemistry. Thank you for providing this exciting topic and also the guidance to manoeuvre through the subject.

I would also like to thank PD Dr. Sascha Beutel for the years of encouragement and trust you showed. It was an honour to defend the final thesis in front of you.

Thank you PD Dr. Ulrich Krings for boosting confidence before the defence and also for being the chairperson of the examination.

This project would not have been a possibility without the support of Sartorius Stedim Biotech GmbH. I owe a debt of gratitude to Dr Oscar-Werner Reif, Dr Gerhard Greller and Sebastian Ruhl.

A huge credit of the work goes to Dr Dörte Solle. Thank you for being my mentor, my guiding light and someone who helped see the “forest and not just the tree”. Over the years I learnt a lot from you and will forever be grateful for everything you taught me.

Thank you Dr Janina Bahnemann for being a friend, a confidant and an inspiration. Your warmth, kindness and intellect has left a memorable impact on me.

Over the years at TCI, several people left a lasting impression on my life. I would like to thank Dr. Antonina Lavrentieva for being the best partner for filling in the liquid nitrogen and also explaining the culture to me, Dr. Iliyana Pepelanova for being a ray of happiness every time we met, Dr. Christian Ude for enlightening me on a number of various topics (I thoroughly enjoyed the talks we shared over the years), Dr. Ivo Havlik for always supporting through any technical problems (hopefully will hear the banjo someday), Martina Weiß and Ulrike Dreschel for being the backbone of TCI without whom we would all be lost. I would also like to thank Kai, Thorleif, Friedbert and Thorsten for all the times you helped me.

This journey was wholesome due to the wonderful colleagues who eventually turned into friends. A huge thank you these people: Jensi, Daniel, Flo, Julie, Katharina, Johanna, Jana, Alina, Marline, Francisco, Basti, Chanti, Lola, Tim, Ina, Marek, Harsh.

Thank you to all the TCI’ers for a wonderful journey.

A special thank you to my parents: Mr Sunil Nagraik and Mrs. Anita Nagraik; my guiding light: Mr Amit Sharma and Mrs Mamta Sharma; my sister Arzoo and my friends Sushree and

Mangalam for your support and patience. You inspired me to believe in myself and motivated me to take on different challenges in life. I will forever be in your debt.