

American University in Cairo

AUC Knowledge Fountain

Theses and Dissertations

6-1-2014

Identification of the critical cultivation parameters for scaling up the upstream process of recombinant human Follicle Stimulating Hormone production from Chinese Hamster Ovary cells

Momen Mohsen Amer

Follow this and additional works at: <https://fount.aucegypt.edu/etds>

Recommended Citation

APA Citation

Amer, M. (2014). *Identification of the critical cultivation parameters for scaling up the upstream process of recombinant human Follicle Stimulating Hormone production from Chinese Hamster Ovary cells* [Master's thesis, the American University in Cairo]. AUC Knowledge Fountain.

<https://fount.aucegypt.edu/etds/1190>

MLA Citation

Amer, Momen Mohsen. *Identification of the critical cultivation parameters for scaling up the upstream process of recombinant human Follicle Stimulating Hormone production from Chinese Hamster Ovary cells*. 2014. American University in Cairo, Master's thesis. AUC Knowledge Fountain.

<https://fount.aucegypt.edu/etds/1190>

This Thesis is brought to you for free and open access by AUC Knowledge Fountain. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of AUC Knowledge Fountain. For more information, please contact mark.muehlhaeusler@aucegypt.edu.



School of Sciences and Engineering

**Identification of the Critical Cultivation Parameters for Scaling
up the Upstream Process of Recombinant Human Follicle
Stimulating Hormone Production from Chinese Hamster
Ovary Cells**

A Thesis Submitted to Biotechnology Master's Program
In partial fulfillment of the requirements for the degree of Masters of Science

By:

Momen Mohsen Amer

Under the Supervision of:

Dr. Asma Amleh, Assistant Professor, AUC.

Dr. Jan Rohde, R&D Manager, Rhein Minapharm Biogenetics.

The American University in Cairo

May 2014

Dedication

To Hosneya H. Amer (1950-2011), my mother, I know how proud you would have been now. To my father, Yasmine, Maryam and Yassin.

Acknowledgments

“That is the Grace of Allah; He brings it to whomever He decides; and Allah is the Owner of the Ever-Magnificent Grace”

I would like to acknowledge the R&D and production departments at Rhein Minapharm Biogenetics; Dr. Jan Rohde, R&D manager, for his guidance and for his continuous support even at the hardest times. Susann Koch, production manager, the godmother of this work.

I would also acknowledge Shaymaa El-Taieb, who contributed in some of the large scale upstream processing runs, Shady Nabil who assisted in some of the large scale as well as lab scale upstream processing, Ahmad Samir Youssef and Alaa Ashraf for assisting in the lab scale upstream processing. Ahmed also assisted in ELISA analysis. Sandy Ezzat and Sylvia Raouf performed the ELISA analysis for the large scale experiments and trained me to perform the lab scale ones. Maria El-Ghazaly developed the method for FSH analysis on HPLC. And Ebram Beshara performed the analysis. Thanks to the technicians, Catherine Asham, Bishoy Nabil in the production area, Demiana Shafiq and Refka Salah in the R&D labs for making everything easier.

I would thank Dr. Asma Amleh for her support, and her amazing understanding how things go with a full time working student.

All gratefulness goes to my mother who built the passion of science and knowledge inside me. I miss you. And to my father, who is always there for me.

All warm and deep appreciations to Yasmine, thank you just for being who you are. Nothing could have been done without you.

I would recognize Dr. Hussein El Sawalhy, who first taught me science and research.

Many thanks to all my colleagues at Rhein Minapharm and at AUC for being so helpful and co-operative.

Abstract

Biotechnology industry has experienced a massive development in the production of recombinant protein therapeutics over the past few decades. Stainless steel bioreactors have been considered the gold standards for the upstream processing for a long time. However, over the past decade, the single use technology has gained a big interest in the field especially with the mammalian and insect cell cultures. The single use based processes offered many advantages over the conventional ones. Disposable systems eliminated the need for cleaning in place (CIP), sterilization in place (SIP) and cleaning validation, reduced the risks of cross contamination, and they also offered less production turnaround times. In 2010, Rhein Minapharm Biogenetics, a leading company in biopharmaceutical production in the Middle East, presented the first single use technology based upstream process for recombinant human- follicle stimulating hormone (rh-FSH) production from Chinese hamster ovary (CHO) cells on the HyClone 50L single use bioreactor (SUB). The process was then scaled up to the BIOSTAT STR 200L bioreactor. However, although the BIOSTAT STR 200L bioreactor offered a higher cell density, the product concentration measured by ELISA was only two thirds, the concentration on HPLC was about 75% and the specific cell productivity was only about 50% when compared to the HyClone 50L SUB process. In the current study, the operational parameters that were observed to be different in the two processes were examined. The aim was to determine the critical cultivation parameters to be considered during the scaling of the process. Those parameters were tested at different operating values covering a wide but reasonable range. The experiments were run on the DASGIP lab scale bioreactor at a 750mL working volume. The tested parameters were the culture cell density, the carbon dioxide partial pressure ($p\text{CO}_2$) and osmolality levels in the culture, and the effect of operating at as much as 10 folds higher power input per unit volume (P/V), the parameter which was also tested on the 200L scale. The experiments on the lab scale showed that the lower cell density and the higher $p\text{CO}_2$ and osmolality levels, being comparable to those of the 50L process yielded results comparable to the HyClone 50L SUB process. On the other hand, operating

at higher cell density and lower pCO₂ and osmolality levels, being comparable to those of the 200L process, yielded results comparable to the BIOSTAT STR 200L process. No remarkable differences were observed either in the cell growth rate, the product titer or in the cell productivity when operating at P/V of ten folds difference. It was concluded that the cell density and the pCO₂ and/or osmolality levels are the critical cultivation parameters in the lab scale experiments. These parameters are to be on focus and to be carefully optimized during scaling up of the process.

Table of contents

Dedication	ii
Acknowledgments	iii
Abstract.....	iv
Abbreviations	viii
List of tables	x
List of figures.....	xi
Chapter1: Introduction	1
1.1 Biotechnology industry and single use technology.....	1
1.2 Scale-up parameters for bioreactors	4
1.2.1 Reactor geometry	5
1.2.2 Mixing time.....	5
1.2.3 Reynolds number (Re).....	6
1.2.4 Tip speed	6
1.2.5 Power input per volume (P/V)	7
1.2.6 Volumetric gas flow rate per unit volume of liquid (Q_g/V) and superficial gas velocity (v_s)	8
1.2.7 Volumetric oxygen mass transfer coefficient (k_La)	8
1.3 Follicle stimulating hormone therapeutic preparations	10
1.4 Purpose of the study.....	11
Chapter 2: Materials and Methods.....	13
2.1 Materials and reagents	13
2.2 Equipment	14
2.3 Methods.....	15
2.3.1 Cell line	15
2.3.2 Upstream process	15
2.3.3 Evaluating the engineering characteristics of the bioreactors	20
2.3.4 Viability and viable cell density	21
2.3.5 Metabolites concentration, pH, pCO_2 , and osmolality analysis.....	22
2.3.6 FSH quantification via ELISA.....	22

2.3.7	FSH quantification via HPLC.....	22
2.3.8	Calculation of specific cell productivity	23
2.3.9	Statistical analysis	23
Chapter 3:	Results.....	24
3.1	Evaluating engineering parameters	24
3.2	Power input per unit volume (P/V)	24
3.3	Mass transfer coefficient (k_{La}).....	25
3.4	Viability and viable cell density	26
3.5	FSH quantification via ELISA.....	31
3.6	FSH quantification via HPLC.....	36
3.7	Cell specific productivity	40
3.8	pCO ₂ level analysis	44
3.9	Osmolality level analysis.....	44
Chapter 4:	Discussion	46
4.1	Effect of common engineering scale up parameters	46
4.2	Effect of cell density	47
4.3	pCO ₂ and osmolality	49
Chapter 5:	Conclusions and Future Recommendations.....	52
5.1	Conclusions.....	52
5.2	Future Recommendations	52
References:	54

Abbreviations

A_v :	Bioreactor inside cross sectional area
C^* :	Saturated dissolved oxygen concentration
ccm:	Cubic centimeter per minute
CHO:	Chinese hamster ovary
CIP:	Cleaning in place
C_L :	Concentration of dissolved oxygen inside the liquid media
D:	Bioreactor diameter
d:	Impeller diameter
D0:	Day of inoculation
D1-D9:	Day number of cultivation process
dhfr:	Dihydrofolate reductase
DO:	Dissolved oxygen
h:	Bioreactor height
h_1 :	Liquid height inside the bioreactor
K', α, β :	Empirical coefficients for calculation of k_{La}
k_{La} :	Volumetric oxygen mass transfer coefficient
Lpm:	Liters per minute
MAb:	Monoclonal antibody
N:	Impeller number of rotations per second
P/V:	Power input per unit volume
P:	Passage number
pCO_2 :	Carbon dioxide partial pressure
P_g :	Power input under aerated conditions
pH _i :	Intracellular pH
PI:	Propidium Iodide
P_o :	Impeller power number
P_{og} :	Impeller power number under aerated conditions
Q_g :	Volumetric gas flow rate

Re:	Reynolds number
rh-FSH:	Recombinant human follicle stimulating hormone
rpm:	Rotation per minute
SIP:	Sterilization in place
SUB:	Single use bioreactor
TFA:	Trifluoroacetic acid
TMB:	3,3',5,5'-Tetramethylbenzidine
TNFR:Fc:	Tumor Necrosis Factor Receptor:Fc Fusion Protein
tPA:	Tissue plasminogen activator
v/v:	Volume per volume
V:	Bioreactor working volume
v:	Superficial gas velocity
VCD:	Viable cell density
vvm:	Gas volume per liquid volume per minute
W/W:	Weight per weight
WCB:	Working cell bank
WFI:	Water for injection
ρ_L :	Liquid density
η_L :	Liquid viscosity

List of tables

Table 1 Engineering parameters for the HyClone 50L SUB and the BIOSTAT STR 200L bioreactor.	24
Table 2 Calculated P/V values of the HyClone 50L SUB and the BIOSTAT STR 200L bioreactor at their in-house established operating conditions.	24

List of figures

Figure 1 Stirred tank bioreactor.	3
Figure 2 Flow Chart for the seed train of the upstream process in the HyClone 50L SUB, BIOSTAT STR 200L and DASGIP 750mL bioreactors.	19
Figure 3 A plot for the change in DO versus time for measuring the k_{LA} in the HyClone 50L SUB at the maximum and the minimum oxygenation rates during the production phase of the process.	25
Figure 4 Average VCD and viability percent of the HyClone 50L batches and BIOSTAT STR 200L batches performed at a P/V ten folds that of the HyClone.....	26
Figure 5 Average VCD and viability percent of the BIOSTAT STR 200L batches performed at ten folds and at equivalent P/V of the HyClone 50L bioreactor.	27
Figure 6 Average VCD and viability percent of the DASGIP 750mL batches performed at different starting cell densities..	28
Figure 7 Average VCD and viability percent of the DASGIP 750mL batches performed at different CO ₂ concentrations.....	29
Figure 8 Average VCD and viability percent of the DASGIP 750mL batches performed at the in-house established operating conditions, at a lower cell density, at 10% CO ₂ containing sparged air, and at both conditions combined.....	30
Figure 9 Average VCD and viability percent of the DASGIP 750mL batches performed at the in-house established operating conditions, and at P/V ten folds higher.	31
Figure 10 Average FSH concentration measured by ELISA the HyClone 50L bioreactor, the BIOSTAT STR 200L bioreactor at its normal working conditions with ten folds P/V more than that of the HyClone and at P/V equivalent to that of the HyClone	32
Figure 11 Average FSH concentration measured by ELISA on the DASGIP 750mL bioreactor at different starting cell densities.	33
Figure 12 Average FSH concentration measured by ELISA on the DASGIP 750mL bioreactor different CO ₂ concentrations..	34

Figure 13 Average FSH concentration measured by ELISA on the DASGIP 750mL bioreactor, at in-house established operating conditions, at a lower inoculation cell density, at 10% CO ₂ containing sparged air, and at both conditions combined	35
Figure 14 Average FSH concentration measured by ELISA on the day of harvest at the DASGIP bioreactor controls, the BIOSTAT STR 200L bioreactor operated at 1X and 10X P/V values, the DASGIP bioreactor at 10% CO ₂ containing sparged air and low inoculation cell density, and at HyClone 50L SUB bioreactor	35
Figure 15 Average FSH concentration measured by ELISA for DASGIP 750mL batches performed at the in-house established operating conditions, and at P/V ten folds higher.	36
Figure 16 Average FSH concentration measured by HPLC on day of harvest for HyClone 50L, BIOSTAT STR 200L operated at P/V ten folds greater than that of the HyClone bioreactor, at P/V equivalent to that of the HyClone and DASGIP controls. ...	37
Figure 17 Average FSH concentration measured by HPLC on the day of harvest for the DASGIP 750mL bioreactor at different starting cell densities.....	38
Figure 18 Average FSH concentration measured by HPLC on the day of harvest for the DASGIP 750mL bioreactor at different CO ₂ concentrations.....	38
Figure 19 Average FSH concentration measured by HPLC on day 9 of the cultivation process for the DASGIP 750mL bioreactor, at in-house established operating conditions, at a lower inoculation cell density, at 10% CO ₂ containing sparged air, and at both conditions combined.	39
Figure 20 Average cell specific productivity for HyClone 50L batches, BIOSTAT STR 200L operated at P/V ten folds greater than that of the HyClone bioreactor, at P/V equivalent to that of the HyClone, and DASGIP controls	40
Figure 21 Average cell specific productivity calculated for the DASGIP 750mL bioreactor at different starting cell densities.	41
Figure 22 Average cell specific productivity on the DASGIP 750mL bioreactor at different CO ₂ concentrations.	42

Figure 23 Average specific cell productivity at in-house established operating conditions, at a lower inoculation cell density, at 10% CO₂ containing sparged air, and at both conditions combined 43

Figure 24 Average cell specific productivity for the DASGIP 750mL bioreactor, at in-house established operating conditions, at both low cell density and 10% CO₂ containing sparged air, for the HyClone 50L SUB, and for the BIOSTAT STR bioreactor 43

Figure 25 Average pCO₂ level in the BIOSTAT STR, DASGIP controls, HyClone SUB, and DASGIP operated at 10% CO₂ containing sparged air. 44

Figure 26 Average Osmolality in the BIOSTAT STR, DASGIP controls, HyClone SUB, and DASGIP operated at 10% CO₂ containing sparged air 45

Chapter1: Introduction

1.1 Biotechnology industry and single use technology

Since 1982, when the first recombinant human insulin was introduced, the market of recombinant protein therapeutics has been growing. Until 2008 FDA approved more than 130 molecules for clinical use and at present there are more than 170 proteins and peptides in use (Żerek & Różga, 2012). This growth is partly attributed to the increase in reliable scale up technologies for culturing mammalian cells, where many issues have been resolved like cells adaptation to suspension culture, shear sensitivity and oxygen supply (Chu & Robinson, 2001). Nonetheless, cultures must be maintained at much larger volumes in order to produce the desired amount of product for industry. Cultivation of cells should be performed at large volumes, and while scaling up to these industrial large volumes is usually associated with changes in the geometric and physical conditions of the culture, such changes might lead to decreased yields and reduced consistency between batches (Schmidt, 2005).

Conventional bioreactors in industry are typically stainless steel units. Several drawbacks for the use of such bioreactors were identified, of which are the need of continual sterilization, the reduction of the overall production time due to required cleaning time between batches, and the increased risk of cross contamination between different cell lines or proteins produced in the same bioreactor. These drawbacks have led to the increased use of single use technology over the past few years, especially with the increased need to deliver high quality products within strict timelines (Forgione & Van Trier, 2006). Single use bioreactors have been successfully scaled up to 2000L working volume for mammalian and insect cell cultures. While for microbial fermentations, disposable systems still pose significant engineering challenges. These challenges are attributed to the fact that in aerobic microbial fermentations, the oxygen demand and the rate of heat evolution are 25-70 times higher than the mammalian and insect cell cultures. Thus, a much faster oxygen supply and heat removal from the culture is required. This was met by the glass and

stainless steel bioreactors but not yet fully met by the disposable plastics at large volumes (Gallagher et al., 2011).

However, there are still some key challenges facing the single use technology developers and end users. These challenges include the limitation in the scale to the current maximum of 2000L in the upstream process, the restricted diversity due to limited number of vendors offering single use equipment, the absence of universal regulations and standardization of the materials used in manufacturing, and hence the increased risk of leachables and extractables from the disposable plastics that could potentially contaminate the product intermediates (Shukla & Gottschalk, 2013).

Single use bioreactors are plastic bags presterilized by their suppliers and thus, sterilization and cleaning are not required. This eliminates the need of large space and costs of cleaning in place (CIP) and sterilization in place (SIP) installations. In the mean time, these single-use systems reduce risks of cross contamination and production turnaround times as they allow companies to shift between cell lines and target proteins in a production process quickly and inexpensively (Genetic Engineering and Biotechnology News, 2006), and with less validation procedures this will definitely shorten time to market (De Wilde et al., 2009).

The benefits of implementing the disposable technology were summarized by a development manager at Sartorius Stedim Biotech, a leading international supplier for single use bags and bioreactors: “Benefits of flexible bag containers include faster facility set-up, reduction of down time, simplified validation, and more efficient use of plant floor space. Disposable bags greatly reduce the risk of cross contamination” (Genetic Engineering and Biotechnology News, 2006).

The disposable systems are polyethylene bags designed to be inserted into stainless steel casings to serve as a bioreactor. After each batch, the bag is removed and a new presterilized bag can be installed for the next one. Two main types of single use bioreactor systems are currently available; wave reactors and stirred reactors. The wave disposable

bioreactors were developed in the 1970s (DiBlasi et al., 2007). They are pillow shaped bags that are placed on a platform. A rocking motion that creates waves provides mixing. Wave bags are more useful at the laboratory scale, while being awkward and space consuming at large scales. All the wave bag components are designed and manufactured to be single use and disposable, except for the control unit and the rocking platform. This includes the bag chamber, vent filters, probes, as well as other tubing and fittings that come in contact with the product. One of the drawbacks of these systems is that they lack the common geometry with the stirred tank reactors, which are the systems of choice in industry (Nienow, 2006).

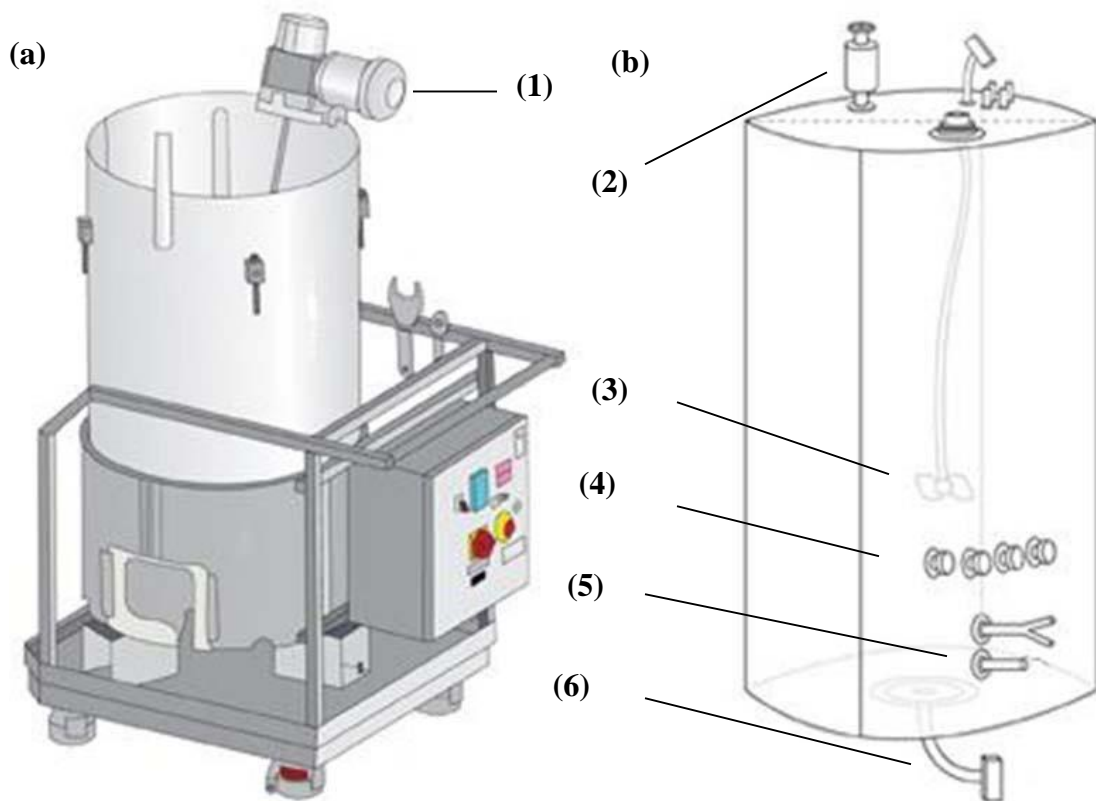


Figure 1: Stirred tank bioreactor: Steel jacket (a) and disposable bag insert (b). Where: (1) Impeller motor and drive shaft, (2) Air filter, (3) Integrated impeller, (4) Probe ports (5) Temperature sensor port and sample tubes, (6) Sparger port. Image from Thermo Fisher website: <<http://www.thermoscientific.jp/hyclone-bpc/catalog/single-use-bioreactor.html>>.

The second type of single use bioreactors, the stirred bags, serves as a typical stirred tank bioreactor and was first introduced in 2006 by ThermoFisher Scientific (Selker & Paldus, 2008). A disposable bag (Figure 1b) is used as a liner in a cylindrical steel tank (Figure 1a). The bag has an integrated impeller for mixing, sampling ports, probe ports, vent filters, feed and harvest tubings, and a sparger system (Figure 1b).

1.2 Scale-up parameters for bioreactors

The biotechnology industry has continued to grow as new protein therapeutics are approved to enter the market (Chu et al., 2001). With the continuous growth of biotechnology industry, the need for a successful scale up strategies also increased. A quicker and more efficient transfer of the laboratory processes to the industrial scale is required. The difficulty with the scale-up comes from the fact that large tanks are much more heterogeneous than small ones (Shuler & Kargi, 2002). And although similar geometry is recommended between the different scales for easier scaling, it remains impossible to maintain the same level of shear, mixing time, and mass transfer from the small to the larger bioreactor since power and aeration requirements do not scale linearly (Stoker, 2011).

There are certain “rules of thumb” that are applied to the scale-up of bioreactors (Catapano et al., 2009). These rules assume that some criteria, when optimized on the small scale, can also be considered optimal at the large scale. These criteria are concerned with either the mass transfer and mixing or the mechanical cell damage. It is impossible to keep all parameters constant across the scales. Maintaining a specific set of parameters constant will change another set of parameters, which can thus produce undesired effects on the behavior and the yield of the culture (Ju & Chase, 1992). So scaling up is often based on the combination of multiple cultivation characteristics. Given the various challenges involved, knowledge and experience are nearly as important as the scale up parameters (Ma et al. 2006). The operator should determine the most critical parameters in his small scale process to be kept constant during the scale up.

The main process parameters that have been suggested in literature to be maintained constant during scale-up include:

1. Reactor geometry
2. Mixing time
3. Impeller Reynolds number, (Re)
4. Tip speed
5. Power input per unit volume of liquid, (P/V)
6. Volumetric gas flow rate per unit volume of liquid, (Qg/V)
7. Superficial gas velocity, (v)
8. Volumetric oxygen mass transfer coefficient, (k_{La})

1.2.1 Reactor geometry

Reactor geometry can be described by the height to diameter ratio (h/D), also known as the aspect ratio, as well as the ratio of the impeller to vessel diameters (d/D) (Stoker, 2011). For stirred tank bioreactors, the aspect ratio is typically between 1:1 and 3:1 (De Wilde et al., 2009).

Bioreactors with low aspect ratios offer a better gas transfer through the head space, while those of higher aspect ratios provide better dispersion and longer residence times for gas bubbles in the culture liquid which will improve gas transfer rates when direct sparging is applied for oxygenation (De Wilde et al., 2009).

In general, tank geometry and hardware, like the impeller or sparger types for instance, are not subjected to change during scale-up. So, the focus is on defining appropriate agitation and gassing conditions to achieve consistent performance.

1.2.2 Mixing time

Mixing time is defined as the time required to reach 95% homogeneity in a mixed vessel upon addition of a substance like feed or base (Menisher et al., 2000). Mixing time is thus important to ensure that these additives mix in a timely manner. This way local regions of

high or low pH and/or nutrients are not formed (Langheinrich & Nienow, 1999; Bylund et al., 1998). Proper mixing time also ensures adequate oxygen delivery to the cells.

Simplest methods to measure the mixing time in a bioreactor are the decolorization and the concentration methods (Noack et al., 2010b). The decolorization method requires a dye to be put into the liquid in the bioreactor in advance. Addition of a decolorizing agent makes the color change or even let it disappear. The time between the addition of the decolorization agent and the entire color change or decolorization is measured, and it represents the mixing time for an entirely mixed fluid. Another method is the concentration method where the conductivity of distilled water is measured following an addition of phosphate buffer at different positions, and the mixing time will be the time taken for the readings to become constant.

1.2.3 Reynolds number (Re)

The Reynolds number (Re) is used to describe the flow regime and turbulence phenomena in stirred bioreactors. A complete turbulent flow and hence an efficient mixing is achieved when $Re > 10000$ (Armenante & Chang, 1998).

Re is calculated by the equation:

$$Re = \rho_L N d^2 / \eta_L \quad \text{Equation 1}$$

Where ρ_L is the density of the fluid in kg/m^3 , N is the impeller number of rotations per second s^{-1} , d is the impeller diameter in m, η_L is the fluid viscosity in kg/m.s . Even at high cell densities the viscosity of the culture suspension media is close to water (Oh et al., 1989).

1.2.4 Tip speed

Impeller tip speed is another agitation related parameter that can be correlated to maximum impeller shear rates. Mammalian cells are more liable to shear damage than microbial cells because they lack a cell wall. Therefore a constant tip speed has been suggested as a scaling criterion for mammalian cell culture agitation. Some guidelines

proposed to keep the tip speed value within the range of 1 and 2 m/s during scale-up (Fenge & Lüllau, 2006). However, many investigators reported that the shear rates required to cause cell damage are much higher than those commonly used in cell culture applications (Ma et al., 2002). As a result, tip speed is not recommended as a primary scaling parameter (De Wilde et al., 2009).

Tip speed is calculated by the equation:

$$\text{Tip speed} = \pi N d \quad \text{Equation 2}$$

1.2.5 Power input per volume (P/V)

Power input per volume, also known as the mean specific energy dissipation rate, is the amount of power transferred to a volume of cell culture through the agitator shaft and impellers. It is the parameter more commonly used for scaling agitation. Mammalian and insect cells cannot handle a lot of power introduced into the culture media as it can shear the fragile cell membranes. The typical range of P/V for the animal cell cultures is 1-50 W/m³, with a global average of 10 W/m³. This is 100-fold lower than the 1000 W/m³ average P/V in microbial fermentations (Rathore et al., 2008). The power input per volume is measured using electrical devices that measure the torque inside the bioreactor (Holland and Chapman, 1966).

The empirical equation used for power input calculation is:

$$P = P_o \rho_L N^3 d^5 \quad \text{Equation 3}$$

Where impeller power numbers, P_o (dimensionless), depend on the agitator type. They are essentially constant for any agitator type, regardless of its diameter relative to the bioreactor diameter, or its speed, and of the bioreactor size, provided geometric similarity is maintained across the scales (Nienow, 1998).

1.2.6 Volumetric gas flow rate per unit volume of liquid (Q_g/V) and superficial gas velocity (v_s)

Volumetric gas flow rate per unit volume (Q_g/V) of liquid means the volume of gas flow, usually measured in liters per minute (Lpm), per bioreactor volume, while superficial gas velocity (v_s) is the volume of gas per cross-sectional area of the vessel.

It is recommended to scale the overall gas velocity separately regarding the minimum and maximum sparger gas flows, where the minimum sparger rate is scaled on constant volumetric gas flow per unit volume per minute, while the maximum sparger rate is scaled on constant superficial velocity (v_s) in order to minimize foaming (Seamans et al. 2008). For animal cell cultivation, the aeration rates should be kept lower than 0.1 vvm (Czermak et al., 2009).

The superficial gas velocity can be calculated from the equation:

$$v_s = \frac{Q_{\text{gas}}}{A_v} \quad \text{Equation 4}$$

Where A_v is the inside cross sectional area of the vessel.

1.2.7 Volumetric oxygen mass transfer coefficient ($k_L a$)

The volumetric mass transfer coefficient is the most commonly applied scale up variable. It includes both agitation and gassing parameters that influence oxygen supply (Alam et al., 2005; Schmidt, 2005; Marks, 2003; Yawalkar et al., 2002). Animal cell cultures require oxygen for growth and formation of product from organic carbon sources. The dissolved oxygen (DO) level is a limiting factor in most fermentation and cell-culture processes. There is always a critical level of DO, above which the oxygen concentration no longer limits growth. Therefore it is always important to keep the DO levels above this critical value throughout the cultivation process. This can be achieved by sparging air or pure oxygen into the bioreactor. For this sparging to be effective, the mass transfer rate of oxygen to the liquid media should be greater or at least equal to the rate of oxygen

consumption by the growing cells. Measurements of k_{LA} ensure that processing conditions are sufficient for an adequate supply of oxygen to the proliferating cells (Kane, 2012).

The four approaches commonly used to measure k_{LA} are the unsteady state, steady state, dynamic and sulfite methods (Shuler & Kargi, 2002). Filling the reactor with water or a culture medium without cells performs the unsteady state method. Then, the reactor is sparged with nitrogen to remove oxygen, which is reintroduced afterwards to the system, while monitoring its level, until it reaches saturation. Plotting the log of changes in oxygen concentration versus time results in a slope which is equal to k_{LA} (Shuler & Kargi, 2002). The ease and simplicity of this method makes it the most commonly used.

The steady state, although it is considered a highly reliable way to measure k_{LA} (Shuler & Kargi, 2002), is difficult to be put into practice. It can be used only when the measurement techniques are highly accurate (Doran, 1995), as it needs the oxygen concentration within the system as well as in all gas exit streams to be measured precisely. This method assumes that the conditions in the culture are in a steady state, and a mass balance on oxygen can calculate the oxygen uptake rate. The k_{LA} will be proportional to the oxygen uptake rate and inversely proportional to the difference of the oxygen concentration at saturation and within the system.

The concept of the dynamic method is similar to the unsteady state method. The only difference is that the dynamic method is done in bioreactors with active cells, thus having the advantage of determining the k_{LA} under actual culture conditions (Stoker, 2011).

The last method, the sulfite method, is based on the idea that the sulfur in the sulfite ion (SO_3^{2-}) is oxidized to sulfate (SO_4^{2-}) in a zero order reaction, when O_2 reacts with Na_2SO_3 , using copper or cobalt ions as a catalyst. The rate of sulfate formation is directly proportional to the rate of oxygen consumption. However, it was noted that this method might overestimate k_{LA} and therefore should be converted to the actual k_{LA} of the system (Van't Riet, 1979).

The formula for calculating k_{La} is:

$$k_{La} = K' \left(\frac{P_g}{V} \right)^\alpha v^\beta \quad \text{Equation 5}$$

Where $P_g = P_{og} \rho_L N^3 d^5$, P_{og} is the power number under aerated conditions. P_{og} is usually less than P_o . However, in animal cell cultures where the air flow rates are very low, P_{og} is considered the same as P_o (Langheinrich et al., 1998). k_{La} also depends directly on v . This equation applies independent of the impeller type and scale. α and β are usually about 0.5 +/-0.1 whatever the liquid is. On the other hand, K' is extremely sensitive to composition. In general, k_{La} in animal cell culture are in the range of 1-15 h^{-1} (Nienow, 2003).

1.3 Follicle stimulating hormone therapeutic preparations

Follicle stimulating hormone (FSH) is a complex heterodimeric glycoprotein secreted by the anterior pituitary gland. It consists of two non covalently linked, non identical protein subunits. The α subunit is composed of 92 amino acids and carries two carbohydrate moieties linked to Asn-52 and Asn-78; the β subunit is composed of 111 amino acids and carries two carbohydrate moieties linked to Asn-7 and Asn-24. The two subunits are not connected by intermolecular covalent bonds. However, each chain contains several intramolecular disulfide bonds which stabilize the molecule tertiary structure. The α subunit is also a part of leutinizing hormone and chorionic gonadotrophin, while the β subunit is specific and unique for this hormone. The FSH present within the anterior pituitary gland exists as a heterogeneous population of different isoforms. These isoforms are identical in their amino acid sequence of the two peptide subunits as well as the attachment points of the carbohydrate side chains. However, the difference between the isoforms lies in the composition of the carbohydrate side chains themselves. The chains can exist in many branched forms which may or may not be capped by sialic acid residues. The less acidic isoforms with few sialic acid residues have shorter half life as they are removed from circulation quickly by binding to the asialoglycoprotein receptor in the liver and kidneys. While the more acidic isoforms which have more sialic acid residues escape capture by the receptor and thus remain for longer periods in the circulation and have greater bioactivity. Due to its complicated tertiary and quaternary structure and

glycosylation this protein requires expression in a eukaryotic system in order to support correct folding and post translational modification.

Human FSH therapeutic preparations are used widely for the treatment of infertility by ovarian stimulation. All pharmaceutical preparations of FSH were initially extracted and purified from urine donated by postmenopausal women. These preparations were proven to be effective and well tolerated. In 1990s recombinant gonadotrophins preparations were introduced and dominated since then (Bassett & Driebergen, 2005). Recombinant human FSH was developed in 1996. This was done by inserting the genes encoding α and β subunits of FSH into expression vectors which were transfected into a Chinese hamster ovary cell line afterwards (Howles, 1996). Later, recombinant FSH was demonstrated to be more cost effective than the urine extracted FSH (Daya, 2002). rh-FSH was also shown to have batch to batch consistency, higher purity, and the potential to overcome production limits (Zwart-van Rijkom et al., 2002).

In 2010, Rhein Minapharm Biogenetics, a leading company for production of recombinant pharmaceutical proteins in Egypt and the Middle East, presented the first cultivation process for CHO cells expressing rh-FSH based on a complete disposable upstream pathway (El Taieb et al., 2010). Recently, the need for scaling up the process to the 200L scale was raised in order to meet the increased market demand.

1.4 Purpose of the study

This study aims to define the critical cultivation and engineering parameters that are to be carefully considered and optimized for a successful scale up of the upstream process of rh-FSH production from the HyClone 50L SUB to the BIOSTAT STR 200L bioreactor.

The differences in the in-house established operating parameters between the two bioreactors will be identified and considered as the potential critical parameters. The effect of these parameters on the process is examined on lab scale DASGIP bioreactor at a 750mL working volume by running experiments at a wide but a reasonable range of these

parameters. All runs are evaluated in terms of viable cell density (VCD), cell viability, product concentration and specific cell productivity.

As a result of this study, it will be possible to identify some parameters, which are proven to have a remarkable impact on the process at the lab scale bioreactor. These parameters will be considered as the critical cultivation parameters that have to be on focus and to be optimized during the scale up of the process to the 200L scale.

Chapter 2: Materials and Methods

2.1 Materials and reagents

- Acetonitrile HPLC grade, (Merck)
- CELLSTAR Filter Cap Cell Culture T-Flasks, (Greiner Bio-One)
- Cell line CHO-DG44, clone number T6-3A7-2G7, MI-WCB#1, (ProBioGen AG, Berlin).
- CultiBag RM10, (Sartorius Stedim Biotech)
- CultiBag RM20, (Sartorius Stedim Biotech)
- CultiBag RM50, (Sartorius Stedim Biotech)
- CultiBag STR 200L, (Sartorius Stedim Biotech)
- D-glucose, (Merck)
- DO probe, (Hamilton)
- EX-CELL[®] CHO DHFR⁻ACF media, (SAFC - Sigma)
- FSH enzyme immunoassay test kit, (BioCheck, Inc.)
- FSH in-house quantification reference no.4, (Reference code: CHO1-03-12/OC01-R4)
- FSH Standard Diluent, (BioCheck, Inc.)
- Glass spinners, (Integral BioSystems)
- Hyclone 50L SUB bioprocess container, (Thermoscientific)
- International recombinant FSH standard for immunoassays, (NIBSC)
- Jupiter C18 5 μ m, 300 A poresize, 4.6 x 250 mm, (Phenomenex)
- L-glutamine, (Merck)
- pH probe, (Mettler Toledo)
- Sodium bicarbonate, (Merck)
- Trifluoroaceticacid (TFA), HPLC grade, (Sigma-Aldrich)

2.2 Equipment

- Bioprofile 400 analyser, (Nova biomedical)
- BIOSTAT B-Plus control unit (Sartorius Stedim Biotech)
- BIOSTAT CultiBag RM control unit (Sartorius Stedim Biotech)
- BIOSTAT CultiBag RM20 rocker, (Sartorius Stedim Biotech)
- BIOSTAT CultiBag RM50 rocker, (Sartorius Stedim Biotech)
- BIOSTAT STR 200L outer support container with mixer drive and control unit, (Sartorius Stedim Biotech)
- Centrifuge universal 5424R, (Eppendorf)
- DASGIP 1L bioreactor control unit, (DASGIP, Eppendorf)
- FLUIDO 96 Washer, (Anthos, HVD)
- HPLC LC-2010C HT, (Shimadzu)
- HyClone 50L SUB outer support container with mixer drive and control unit, (Thermoscientific)
- NucleoCounter NC-10, (Chemometec)
- pH meter SevenMulti, (Mettler Toledo)
- Thermomixer comfort, (Eppendorf)
- Zenyth 3100 spectrophotometer, (Anthos, HVD)

2.3 Methods

2.3.1 Cell line

A CHO cell line that is adapted to grow in suspension (CHO-DG44 clone), and transfected with the amplifiable gene construct dihydrofolate reductase (dhfr) was used for transformation with expression cassettes containing the genes encoding the 2 subunits of human follicle stimulating hormone (rh-FSH). This part was performed at ProBioGen AG (Berlin). Cells from a characterized working cell bank (MI-WCB#1) were used in all experiments.

2.3.2 Upstream process

For all scales, the cells were grown in EX-CELL[®] CHO DHFR⁻ACF media (SAFC - Sigma), supplemented with 3 mM glutamine (Sigma). The incubation period between every two passages (P) was 1-3 days. All passages were performed at starting inoculation viable cell densities between 0.30 and 0.60 x 10⁶ cells/mL. T-flasks and glass spinners were incubated at 37°C in 5% CO₂ atmosphere. Wave CultiBags were incubated at 37°C and air sparging at 1Lpm containing 2.5-10% CO₂. In all final bioreactors, the following cultivation parameters were fixed:

- Total batch running time: 9 days.
- Temperature: 37°C reduced to 34°C after 3 days of cultivation.
- Dissolved oxygen set point: 30%, controlled by percent of pure oxygen sparging and air sparger flow rate.
- pH set point: 7.20 (+/- 0.1) controlled with 7.5% w/w sodium bicarbonate solution addition and CO₂ sparging.
- Addition of 30% glucose solution on demand to keep the glucose level in culture above 3 g/L

2.3.2.1 BIOSTAT STR 200L bioreactor

Revitalization of one vial from the characterized WCB was done in 25mL of media in a disposable T-flask. The following two passages were also done in disposable T-flasks

reaching 350-400mL total culture volume. Passages 3 and 4 were performed in CultiBag RM10 wave bag, Sartorius Stedim Biotech, at a culture volume of 1.2L and 4.5L respectively. One day prior passage 5 in the CultiBag RM50, Sartorius Stedim Biotech, 15L of culture media were added to the CultiBag RM50 for media conditioning polarization of the pO₂ probe. On the passage day, a volume of 3.5L fresh media and 4L cell suspension from the CultiBag RM 10L were used for the inoculation of the CultiBag RM50 with the final working volume reaching 22.5L. One day prior to inoculation of the BIOSTAT STR 200L bag, Sartorius Stedim Biotech, 50L of media were added to the STR bag for polarization of the oxygen probe and media conditioning at 37°C for 24 hours. Afterwards, passage 6 was done by transferring the cell culture from the CultiBag RM50 to the BIOSTAT STR 200L bag. One day later, passage 7 was performed by adding fresh culture media to the BIOSTAT STR 200L bag to reach a final volume of 160-180L according to the cell density (Figure 2).

Two batches were performed at agitation speeds starting with 110rpm and increased gradually till reaching 150rpm maximum from day 4 till the end of cultivation. Another batch was performed at agitation speed of maximum 70rpm.

2.3.2.2 HyClone 50L SUB

Revitalization of one vial from the characterized WCB was done in 20mL of media in a disposable T-flask. The following three passages were also done in disposable T-flasks reaching 700 mL total culture volume. Passages 4 and 5 were performed in CultiBag RM10 and CultiBag RM20 wave bags at culture volumes of 2.8L and 8.8L respectively. One day prior to inoculation of the HyClone bag, 17L of media were added to the HyClone bag for polarization of the oxygen probe and media conditioning at 37°C for 24 hours. Afterwards, passage 6 was done by transferring the cell culture from the CultiBag RM20 to the HyClone bag. One day later, passage 7 was performed by adding fresh culture media to the HyClone bag to reach a final volume of 50L (Figure 2). Agitation speed was set at 90rpm and increased gradually every day to reach 120rpm on day 4 till the end of the process.

2.3.2.3 DASGIP 750mL bioreactor

Lab scale experiments on the DASGIP 750mL bioreactor were performed to examine the potential critical cultivation parameters that were identified to show a remarkable difference in their values between the HyClone 50L and the BIOSTAT STR 200L processes.

Three experiments were performed on the DASGIP 750mL bioreactor. Eight vessels were operated in parallel for each experiment, two vessels operated at the normal operating conditions serving as controls, and two sets of triplicates for every test condition.

Revitalization of one vial from the characterized WCB was done in a disposable T-flask in 25mL of media. The following three passages were also done in disposable T-flasks reaching a culture volume of 900mL. Passage 4 was performed in 4 glass spinners with 600mL culture volume in each spinner. One day prior to inoculation of the DASGIP vessels, 500mL of media were added to each of the 8 DASGIP vessels for polarization of the oxygen probe and media conditioning at 37°C for 24 hours. Afterwards, Passage 5 was performed by inoculation of the 8 vessels from the glass spinners (Figure 2).

Different parameters were tested in DASGIP 750mL bioreactor, which were the viable cell density, pCO₂, osmolality in the culture, and P/V. A total of six vessels were cultivated as controls. The control conditions are the normal operating conditions of the DASGIP bioreactor in the plant. The inoculation of the control vessels was done at viable cell densities range of 0.41-0.47 x10⁶ cells/mL. Normal air was sparged into the culture vessel throughout the whole run at the rate of 0.5 Lpm. CO₂ was introduced when needed for pH control. And agitation speed was set in the range of 90-120 rpm reaching its maximum on day 4 of cultivation.

Three vessels for each test parameter were processed. For testing the effect of viable cell density, the low cell density vessels were inoculated at 0.27-0.33 x10⁶ cells/mL while the high cell densities were inoculated at 0.68-0.76 x10⁶ cells/mL. Two test conditions were set for the effect of the pCO₂. This was done by introducing a constant 5% and 10% of CO₂

in the sparged air into the vessel starting from day 1 of the cultivation process till its end. Another set of test vessels were performed by combining the low inoculation cell density with the 10% CO₂ containing sparged air. Processing 3 vessels at a P/V value ten times that of the controls performed testing the effect of high P/V, thus the agitation speed was set at 200 rpm on inoculation and increased gradually to reach 260 rpm on day 4 of the process.

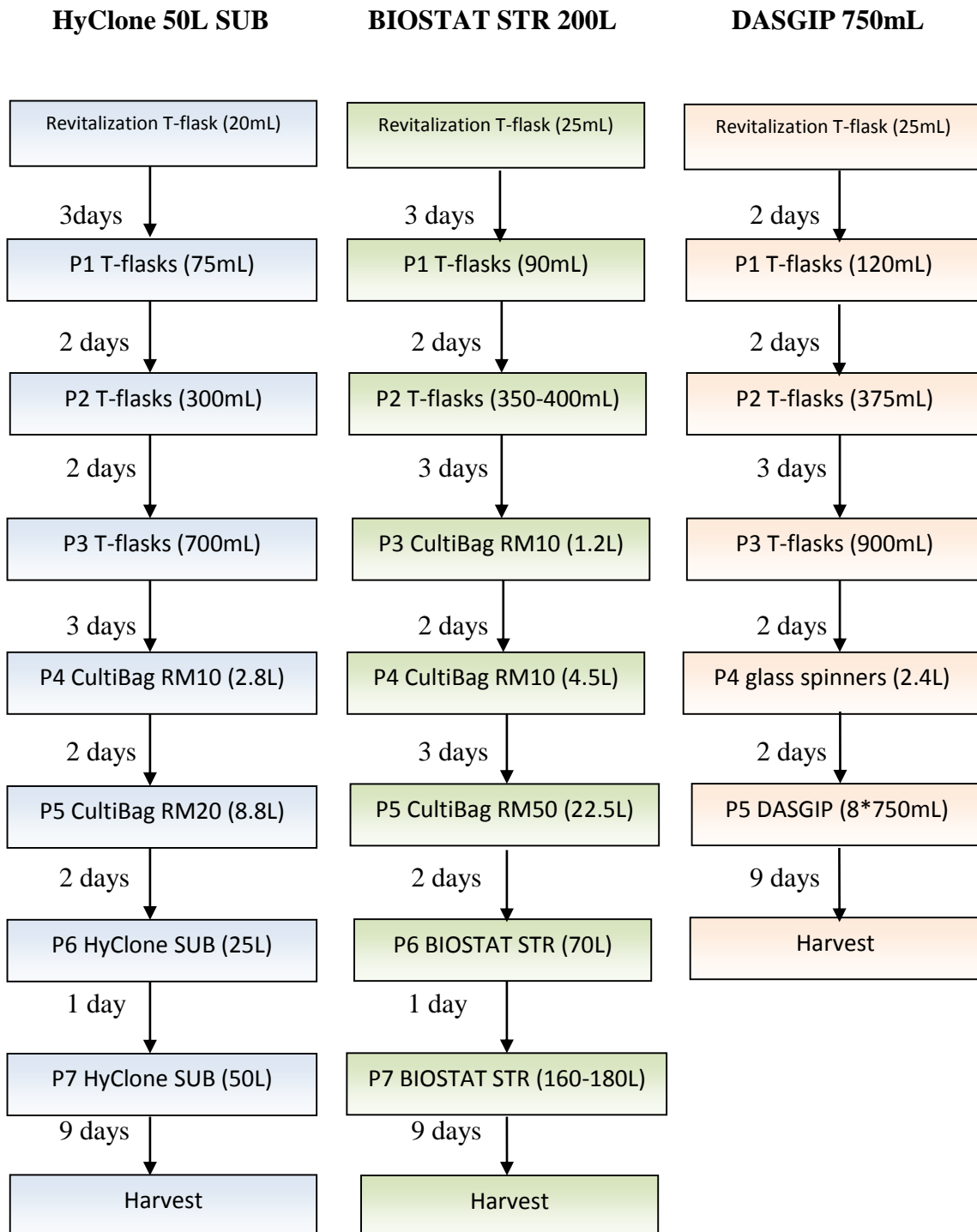


Figure 2: Flow chart for the seed train of the upstream process in the HyClone 50L SUB, BIostat STR 200L and DASGIP 750mL bioreactors.

2.3.3 Evaluating the engineering characteristics of the bioreactors

Evaluation of the engineering characteristics of the mammalian cell bioreactors, HyClone 50L SUB, and BIOSTAT STR 200L, was performed specifically for the geometrical and design aspects important for solving empirical formulas of some critical scale up parameters. These aspects include reactor height, reactor diameter, impeller diameter, impeller type, and power number.

2.3.3.1 k_{LA} measurement

k_{LA} values in the BIOSTAT STR 200L bag were published at different sparging rates and working volumes (De Wilde et al., 2009; Noack et al., 2010a; Noack et al., 2010b). Three of the published values were used to solve the three empirical constants K' , α , and β in equation 5. The calculated values were validated by substitution in the equation to verify the other published k_{LA} values other than the three used for solving the constants. Then, the equation with the validated constants was used to calculate the k_{LA} of the process at the different working agitation speeds during the production phase of the process.

For the HyClone 50L SUB, the k_{LA} was measured by using the unsteady state gassing out method (Wise, 1951). The bag was filled with WFI at 37°C and an agitation speed of 120 rpm was initiated. The DO level was reduced to minimum by purging pure nitrogen gas into the bag. Afterwards oxygenation was performed either at the minimum or the maximum oxygen supply rate during the production phase of the process (from day 4 to day 9). The maximum oxygenation rate was at 60ccm pure oxygen sparging and the minimum oxygenation rate was 53ccm air sparging. The level of dissolved oxygen was monitored via a DO probe, Hamilton, until it reached saturation. k_{LA} was computed by the following equation:

$$\frac{dC_L}{dt} = k_L a (C^* - C_L) \quad \text{Equation 6}$$

Where C_L is the concentration of dissolved O_2 in the fermentation broth, t is time, dC_L/dt is the change in O_2 concentration over a time period, k_{LA} is the volumetric transfer

coefficient in reciprocal time (h^{-1}), and C^* is the saturated dissolved O_2 concentration (100%).

A plot of $\ln(C^* - C_L)$ versus t was done producing a straight line where the slope is equal to k_{La} (Shuler & Kargi, 2002). Only DO values between 10% and 90% were considered (Noack et al., 2010b).

2.3.3.2 Calculation of power input per unit volume (P/V)

The power input per unit volume was calculated for both the HyClone 50L SUB and the BIOSTAT STR 200L bioreactor using equation 3.

The power number (P_o) of the HyClone 50L SUB impeller is 2.2 (Eibl & Eibl, 2009) and 1.3 for the BIOSTAT STR 200L impeller (Noack et al., 2010a). The density, ρ , was considered 1000 kg/m^3 since the cells media suspension is considered water like (Oh et al., 1989). The impeller diameter for the HyClone 50L SUB is 11.75cm (Thermo Scientific HyClone SUB 50L datasheet, website: <https://fscimage.thermoscientific.com/images/D17255~.pdf>) and 22.5cm for the BIOSTAT STR 200L (Noack et al., 2010a).

The required agitation speed to operate the BIOSTAT STR at an equal P/V of the HyClone as well as that required to operate the DASGIP 750mL bioreactor at ten-fold its normal operating P/V were calculated using the same equation.

2.3.4 Viability and viable cell density

Viable cell densities and viabilities were measured by NucleoCounter® NC-100™, Chemometec. The device is an integrated fluorescence microscope designed to detect signals from the fluorescent dye, propidium iodide (PI) bound to DNA.

Results from the NucleoCounter represent either total (the viable plus the non-viable cells) or non-viable cell concentration, depending on the sample preparation. For each sample, by measuring the total and the non-viable cell density, the device calculates the viable cell density and the viability percent.

2.3.5 Metabolites concentration, pH, pCO₂, and osmolality analysis

Samples were withdrawn from each cultivation system daily during the whole 9 days cultivation period. Samples were centrifuged and the supernatants were analyzed for pH, pCO₂, Substrates (glucose and glutamine), and metabolites (ammonia and lactate) concentrations. Analysis was performed on Bioprofile 400 analyser, (Nova biomedical).

2.3.6 FSH quantification via ELISA

FSH concentration was determined using the FSH enzyme immunoassay test kit from BioCheck, Inc. The test was performed as instructed by the manufacturer.

In short, the assay is based on using a mouse monoclonal anti- α -FSH antibody for solid phase (microtiter wells) immobilization and another mouse monoclonal anti- β -FSH conjugated with horseradish peroxidase enzyme. The test sample is allowed to react simultaneously with the antibodies, resulting in FSH molecules being sandwiched between the solid phase and enzyme-linked antibodies. Addition of the chromogen TMB (3,3',5,5'-Tetramethylbenzidine) leads to a color development that is directly proportional to the bound FSH. The spectrophotometric measurement was done on Anthos Zenyth 3100 Microplate Multimode detector at 450nm.

2.3.7 FSH quantification via HPLC

Samples withdrawn on day 9 of cultivation were centrifuged, and aliquots of 100 μ L were analyzed on a Shimadzu LC-2010CHT RP-HPLC at a wavelength of 210 nm. Samples were separated on a Jupiter C18 300A pore size, 4.6 x 250 mm, 5 μ m column, Phenomenex, at a temperature of 35°C. Elution was performed with a gradient of buffer A [0.1% (v/v) TFA] and buffer B [0.1% (v/v) TFA in acetonitrile], over 40 minutes. The flow rate was adjusted to 1mL/min. This method completely dissociates FSH into its 2 chains, α and β , where the α chain elutes in the form of a sharp peak. Due to this complete dissociation, the alpha chain peak area is used as a quantitative representative of the total FSH concentration by profile comparison with an in-house FSH quantification reference (Reference code: CHO1-03-12/OC01-R4).

2.3.8 Calculation of specific cell productivity

The cell specific productivity was calculated by dividing total quantity of the protein measured by ELISA in µg/mL produced during the production phase of the process by the average viable cell count during the same period multiplied by the production phase duration (5 days) to have the specific cell productivity in µg/cell/day (Equation 7).

Specific cell productivity =

$$FSH \text{ concentration (D9)} - FSH \text{ concentration (D4)} / \frac{VCD (D9) + VCD (D4)}{2} \times 5 \text{ (days)}$$

Equation 7

2.3.9 Statistical analysis

The ELISA and HPLC results on Day 9 (day of harvest) as well as the specific cell productivities for the HyClone 50L SUB, BIOSTAT STR 200L, DASGIP controls and the DASGIP operating at both low cell density and 10% CO₂ containing sparged air were compared using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test using GraphPad Prism 5 software. P-values <0.05 were considered significant.

Chapter 3: Results

3.1 Evaluating engineering parameters

The engineering parameters (Table 1) showed that the HyClone 50L SUB bag had a 12% higher liquid height to diameter ratio and impeller to the bag diameter ratio than the BIOSTAT STR 200L bag. The impeller power number of the HyClone 50L SUB was 41% higher than that of the BIOSTAT STR 200L bioreactor.

Table 1: Engineering parameters for the HyClone 50L SUB and the BIOSTAT STR 200L bioreactor.

Parameter	HC 50L bag	STR 200L bag
Reactor height/diameter ratio (h/D)	1.9:1	1.8:1
Liquid height/diameter ratio (h _l /D)	1.5:1	1.34:1
Impeller/bag diameters (d/D)	0.38	0.34
Impeller diameter (d) m	0.1175	0.225
Bag diameter (D) m	0.349	0.585
Impeller type	2 x 3-blade segment impeller	3-blades Pitched blade impeller
Impeller power number (P _o)	2.2	1.3

3.2 Power input per unit volume (P/V)

The P/V results for both the HyClone 50L SUB and the BIOSTAT STR 200L bioreactor result at their in-house established operating conditions (Table 2), indicated that the BIOSTAT STR 200L bioreactor was operating at approximately 10 times higher P/V than the HyClone 50L SUB.

Table 2: Calculated P/V values of the HyClone 50L SUB and the BIOSTAT STR 200L bioreactor at their in-house established operating conditions.

P/V W/m ³				
Days	D1	D2	D3	D4-D9
HyClone 50L bag	3.33	4.59	6.03	7.88
BIOSTAT STR 200L bag	30.6	40.0	56.9	78.1
Ratio STR/HyClone	9.19	8.71	9.43	9.91

3.3 Mass transfer coefficient (k_{La})

Calculating the slopes of the two curves in Figure 3 resulted in the k_{La} values at both the maximum and minimum sparging rates in the HyClone 50L SUB during the production phase of the process. The values were 0.79 h^{-1} and 0.73 h^{-1} . Those values were only about 35% of the 2.27 h^{-1} k_{La} calculated for the BIOSTAT STR 200L bioreactor at its in-house established operating conditions. However, they were about 90% of the 0.86 h^{-1} computed for the BIOSTAT STR 200L bioreactor at an equivalent P/V to the HyClone 50L SUB.

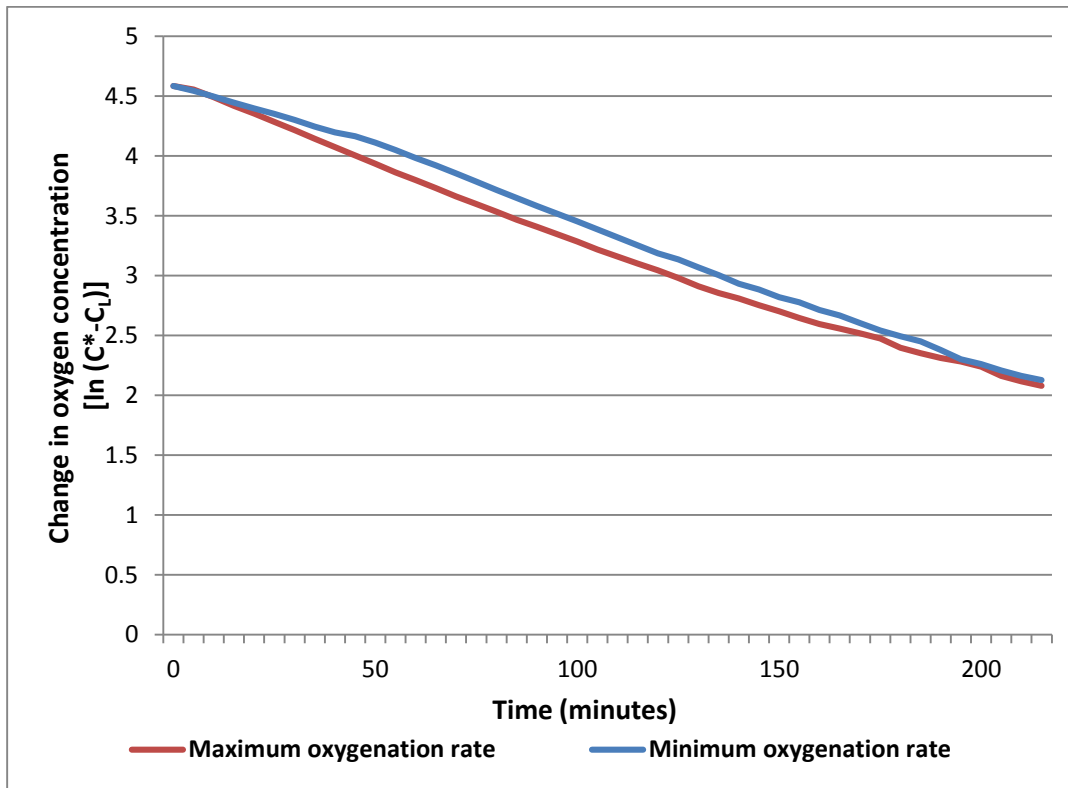


Figure 3: A plot for the change in DO versus time for measuring the k_{La} in the HyClone 50L SUB at the maximum and the minimum oxygenation rates during the production phase of the process.

3.4 Viability and viable cell density

For three batches performed on the HyClone 50L SUB, the maximum viable cell density reached in any batch was 4.31×10^6 cells/mL with an average of 3.96×10^6 cells/mL. The average viability percent on the day of harvest was $86.7\% \pm 1.4$.

While for the BIOSTAT STR 200L bioreactor, the two batches performed on the in-house established working conditions, those conditions representing a P/V value approximately 10 times more than that of the HyClone 50L bioreactor, the maximum cell densities reached for the two batches were 5.35 and 4.70×10^6 cells/mL. The viabilities on the day of harvest were 62.0% and 71.0% . This represented a marked increase in the maximum cell density achieved in the BIOSTAT STR 200L bioreactor compared to the HyClone 50L SUB whose average maximum cell density was only 79% of that reached in average in the BIOSTAT STR 200L. However, the viability percent started to decrease sharply in the BIOSTAT STR 200L batches on day 5 (Figure 4) reaching 20.2% lower values at the day of harvest when compared to the HyClone 50L SUB batches.

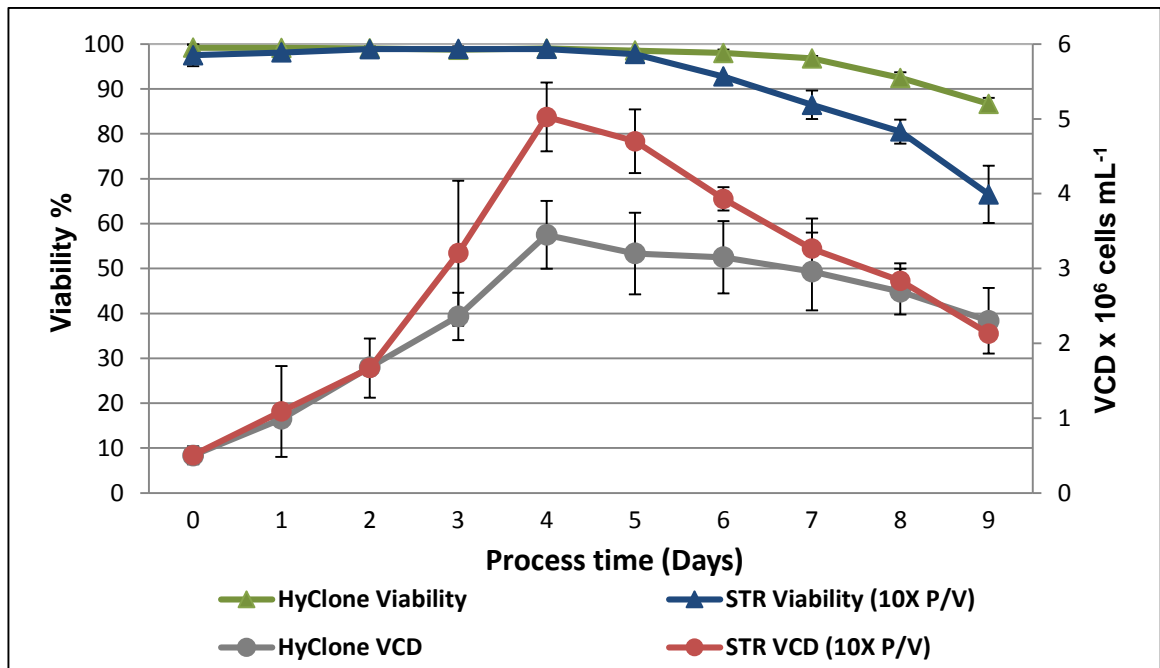


Figure 4: Average VCD and viability percent of the HyClone 50L batches and BIOSTAT STR 200L batches performed at a P/V ten folds that of the HyClone.

The third batch performed on the BIOSTAT STR 200L bioreactor at P/V equivalent to that of the HyClone SUB showed a higher growth rate with an increase in the maximum cell density reaching 5.73×10^6 cell/mL on day 4 of cultivation. The viability percent was highly comparable to the average of the other two BIOSTAT STR batches throughout the process except for the last day of cultivation where the lower P/V resulted in less cell death which was reflected in a 10% higher viability on the day of harvest compared to the average of the two batches operated at the 10 folds P/V (Figure 5).

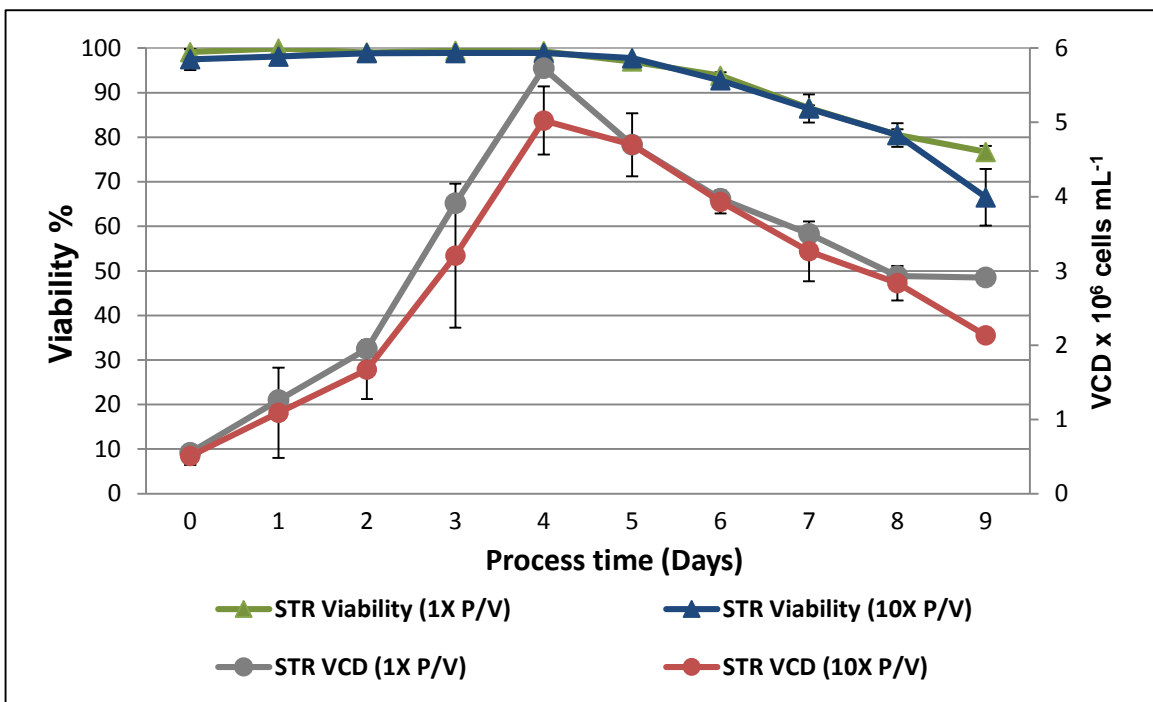


Figure 5: Average VCD and viability percent of the BIOSTAT STR 200L batches performed at ten folds and at equivalent P/V of the HyClone 50L bioreactor.

For the DASGIP 750mL bioreactor, the average of the six control vessels showed a maximum viable cell density of $5.17 \times 10^6 \pm 0.43$ cells/mL and a viability percent on the last day of cultivation of $66.2\% \pm 7.1$.

When starting the cultivation at a higher cell density of $0.68-0.76 \times 10^6$ cells/mL, a higher average maximum cell density $5.72 \times 10^6 \pm 0.26$ cells/mL was achieved, while the viability on the day of harvest was less than the controls, $61.5\% \pm 1.6$. On the other hand, inoculation of the bioreactor at a lower cell density of $0.27-0.33 \times 10^6$ cells/mL than the established

inoculation density of $0.41\text{-}0.47 \times 10^6$ cells/mL lead to a lower average maximum cell density of $3.81 \times 10^6 \pm 0.12$ cells/mL, which is less than the controls by 1.36×10^6 cells/mL, and a viability of $76\% \pm 0.75$ with approximately 10% increase than the controls (Figure 6).

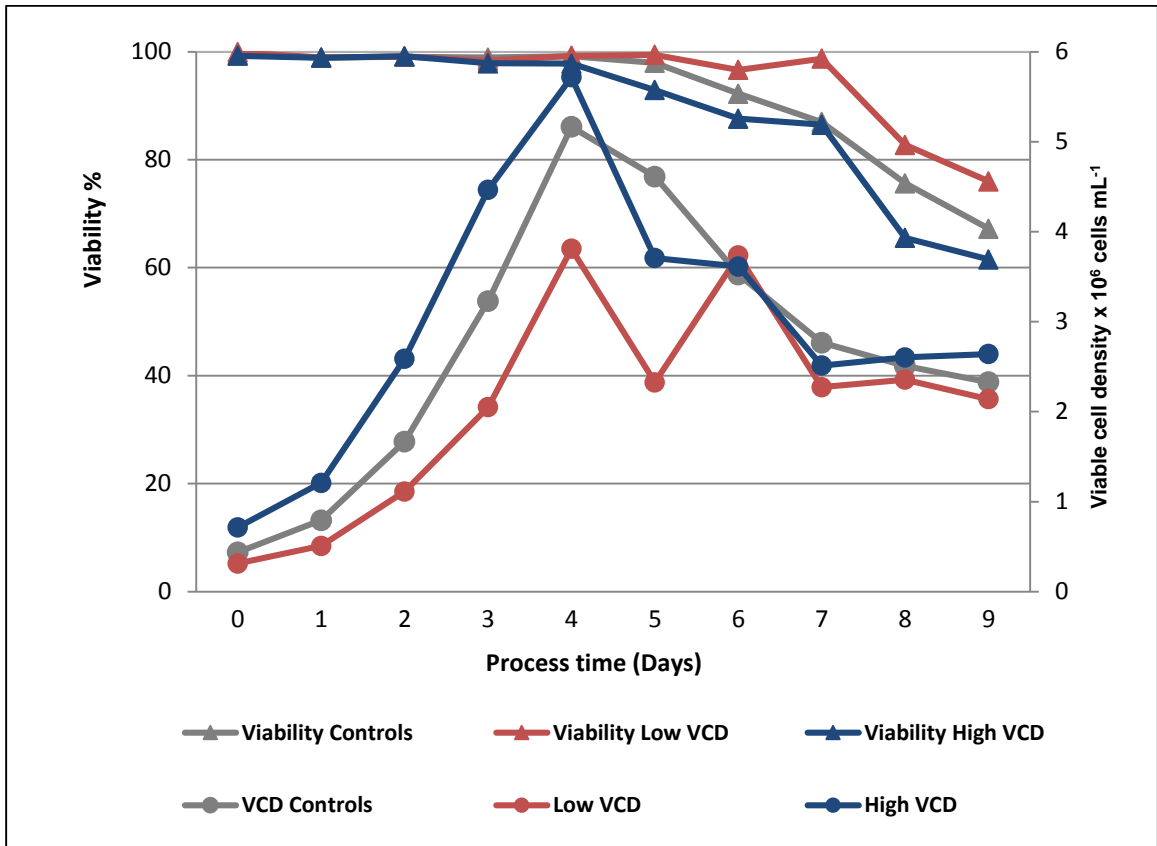


Figure 6: Average VCD and viability percent of the DASGIP 750mL batches performed at different starting cell densities. At the in-house established working conditions (Controls, n=6), at a lower starting cell density, n=3, and at a higher starting cell density (n=3).

For examining the effect of high CO₂ concentration in the culture on the process, purging 5% CO₂ containing air constantly in the culture did not show a remarkable difference in the maximum cell density from the controls, $5.04 \times 10^6 \pm 0.2$ cells/mL, while increasing the percentage of CO₂ in the sparged air to 10% led to a decline in the maximum cell density to an average of $4.52 \times 10^6 \pm 0.09$ cells/mL. The behavior of the viability percent curve was nearly the same with both 5% and 10% CO₂ concentrations in the sparged air. The viability reached $75.5\% \pm 3.8$ and $76.4\% \pm 1.4$ on the day of harvest in the two conditions,

respectively. This is about 10% higher than the controls, which showed a sharper decline in the viability starting from day 6 of cultivation (Figure 7).

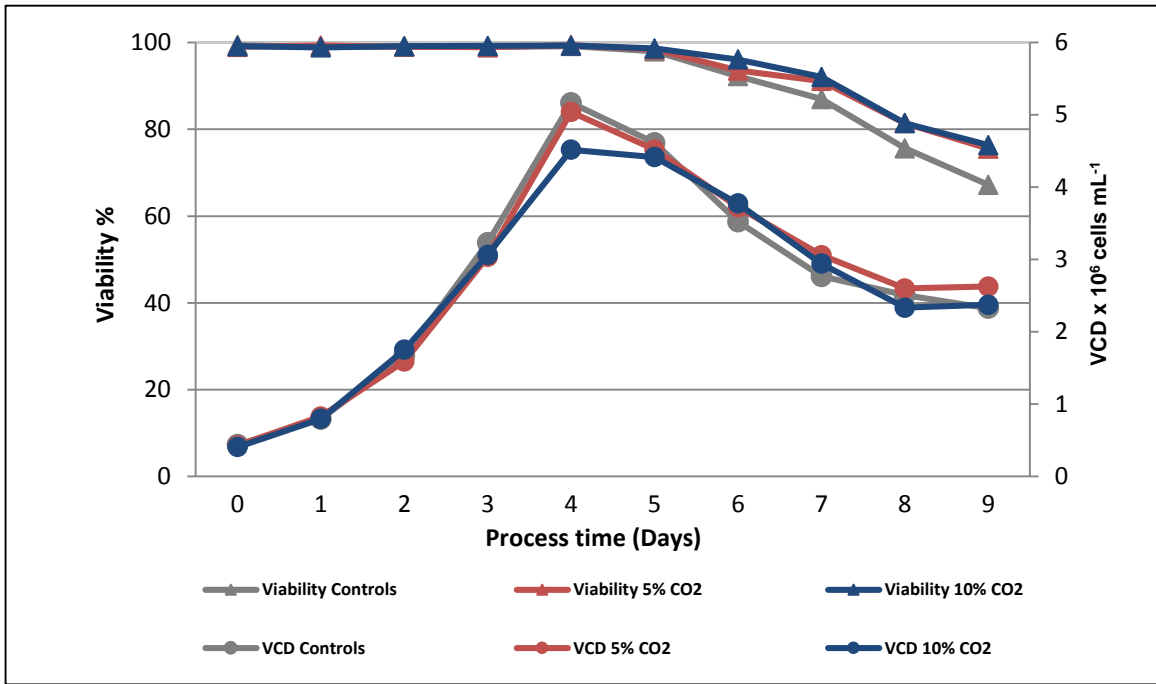


Figure 7: Average VCD and viability percent of the DASGIP 750mL batches performed at different CO₂ concentrations. At the normal working conditions (Controls), n=6, at a 5% CO₂ containing sparged air, n=3, and at 10% CO₂ containing sparged air, n=3.

Combining both conditions of low inoculation density and 10% CO₂ in the sparged air resulted in $3.51 \times 10^6 \pm 0.14$ cells/mL maximum cell density which is the least maximum cell density reached in all tested conditions on the DASGIP bioreactor and also the least viable cell density on the day of harvest. However, the average viability on the day of harvest was $77.8\% \pm 2.22$, which is highly comparable to that observed when each parameter (10% CO₂ and Low cell density) was tested alone (Figure 8).

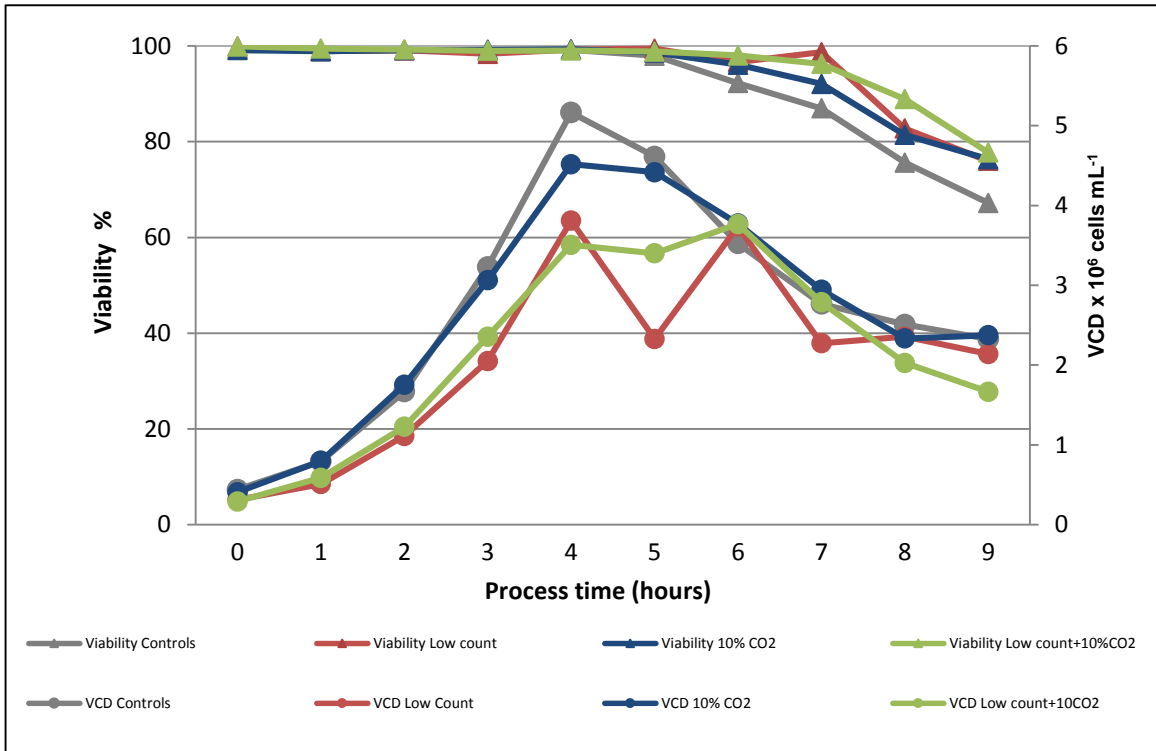


Figure 8: Average VCD and viability percent of the DASGIP 750mL batches performed at the in-house established operating conditions (Controls), n=6, at a lower cell density, n=3, at 10% CO₂ containing sparged air, n=3, and at both conditions combined (Low starting cell density and 10% CO₂ containing sparged air), n=3.

The effect of increasing P/V ten times more than the normal operating conditions of the DASGIP bioreactor was a slight decrease in the viable cell density starting from day 2 of cultivation till the end of the process. The viability percent was highly comparable except for the last day where a sharper decline by 6.9% was observed at the higher P/V (Figure 9).

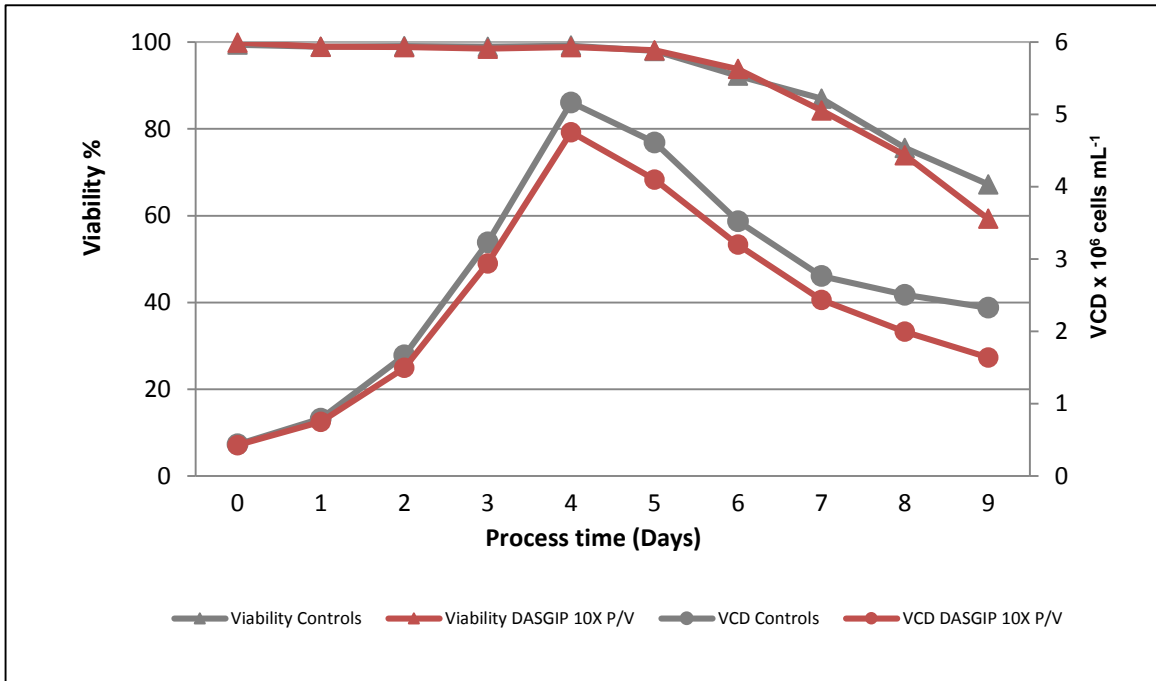


Figure 9: Average VCD and viability percent of the DASGIP 750mL batches performed at the in-house established operating conditions (Controls), n=6, and at P/V ten folds higher, n=3.

3.5 FSH quantification via ELISA

For the HyClone 50L SUB batches, the daily ELISA analysis showed a continuous gradual increase in the average concentration of FSH till the day of harvest. The maximum average concentration measured by ELISA was 407 ± 66 IU/mL on day 9. For the two BIOSTAT STR 200L batches performed at 10 times higher P/V than that of the HyClone 50L SUB, the product concentrations also increased gradually and were highly comparable to the HyClone 50L SUB batches except for the last 2-3 days of cultivation. In those late days, the increase in the product concentration was minimal if any. This resulted in an average concentration from the two BIOSTAT STR 200L batches of 275 ± 16 IU/mL, which represented only two thirds of the average concentration produced from the HyClone 50L SUB batches on the day of harvest.

Reducing the P/V in the BIOSTAT STR 200L bioreactor to the operation value of the HyClone 50L SUB did not show a difference in the trend of the protein concentration throughout the process except for the observation that the increase in concentration

continued till day 9. However the final concentration was nearly the same, 273 IU/mL (Figure 10). Comparing the analysis results of day 9 between the three HyClone 50L SUB runs and the three BIOSTAT STR 200L runs revealed a significant difference with P-value <0.001.

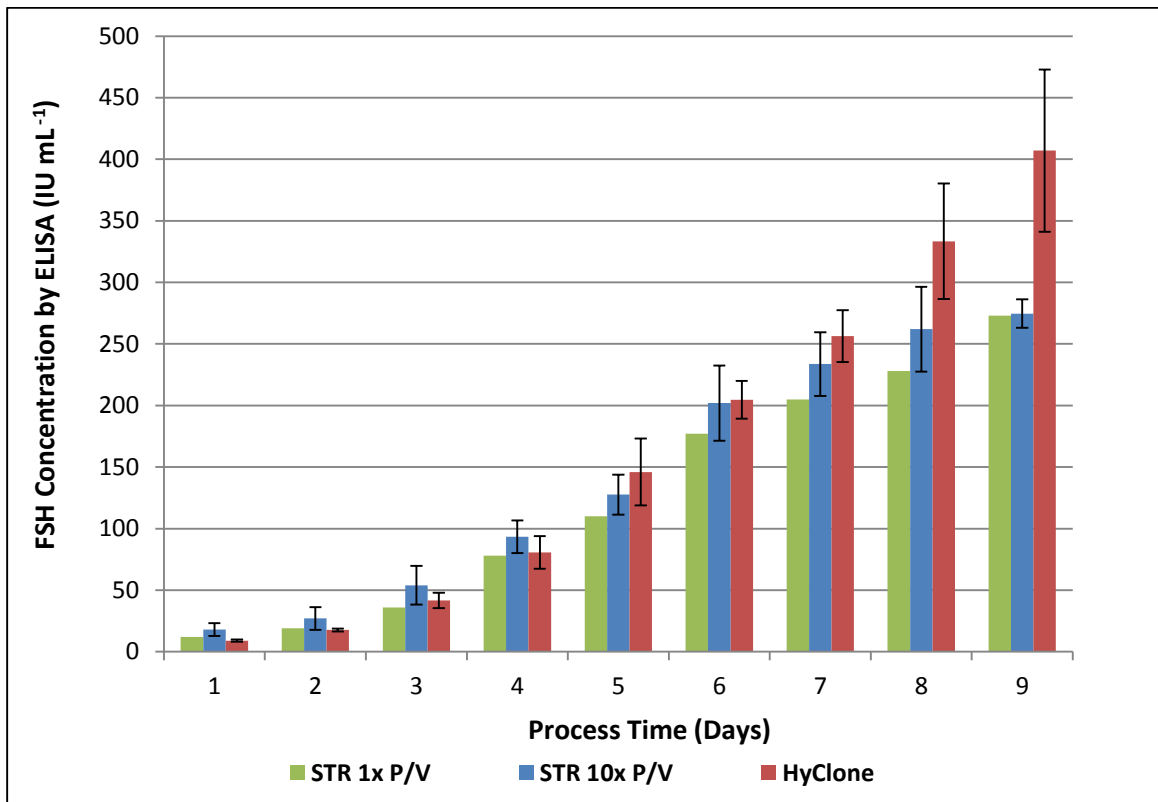


Figure 10: Average FSH concentration measured by ELISA the HyClone 50L bioreactor, n=3, the BIOSTAT STR 200L bioreactor at its normal working conditions with ten folds P/V more than that of the HyClone (STR 10x P/V), n=2, and at P/V equivalent to that of the HyClone (STR 1x P/V), n=1.

For the DASGIP 750mL bioreactor, the controls showed a daily gradual increase in the product concentration average except for the last day where the concentration was nearly the same as that measured on day 8, a behavior which is very similar to that observed in the BIOSTAT STR 200L bioreactor operated at high P/V value. The average concentration on the last day of cultivation was 258 ± 19 IU/mL which is also highly comparable to that produced from the BIOSTAT STR 200L bioreactor (Figure 11). Statistical analysis revealed that the controls analysis results on day 9 were significantly different from the HyClone 50L SUB runs with P-value <0.001, but no significant difference from the

BIOSTAT STR 200L results was shown. At higher inoculation cell densities this concentration was reduced to 217 ± 20 IU/mL. The daily increase in the product concentration did not continue beyond day 6 of the process under those conditions. On the other hand, when starting the cultivation at a lower viable cell density (Figure 11), the average product concentration continued to rise daily throughout the whole process and the final concentration was 304 ± 20 IU/mL, with an 18% increase versus the controls.

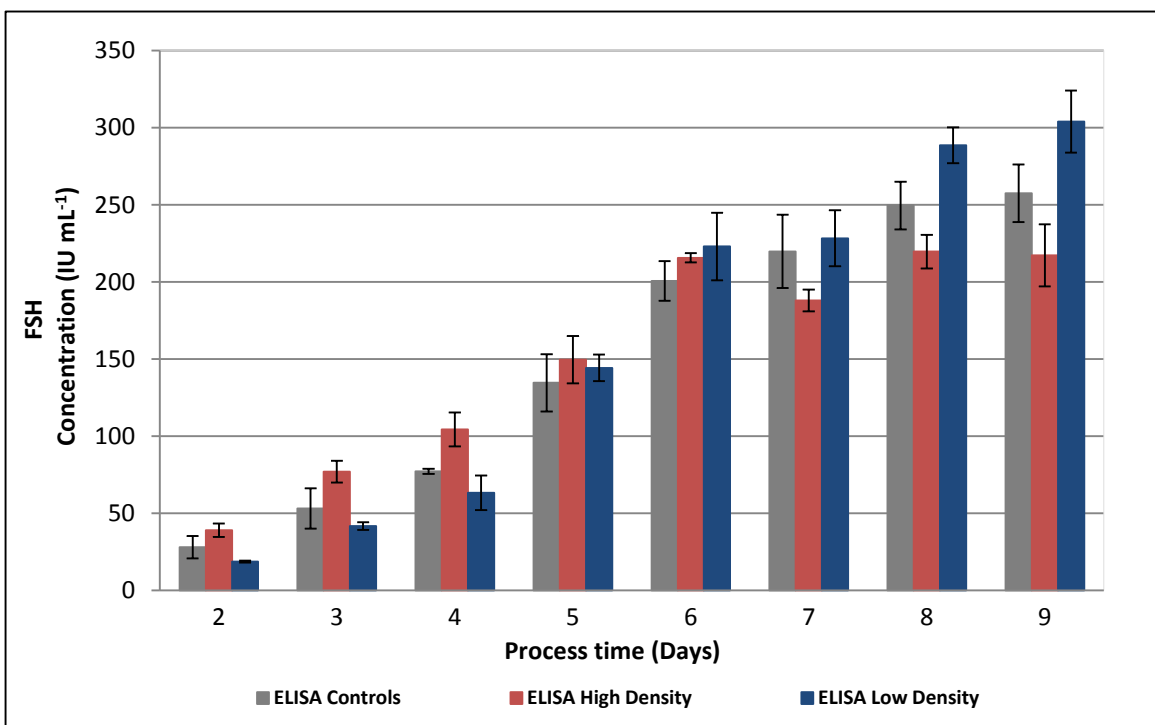


Figure 11: Average FSH concentration measured by ELISA on the DASGIP 750mL bioreactor at different starting cell densities. At normal working conditions (controls) $n=6$, at higher inoculation VCD (High Density), $n=3$, and at lower inoculation VCD (Low Density), $n=3$.

Sparging air containing 5% CO_2 into the culture produced 278 ± 11 IU/mL of FSH on the last day of cultivation. Although the value was comparable to the controls, the concentration was further increased to 325 ± 20 IU/mL when the CO_2 percentage was increased to 10% in the sparged air. This represented a 26% increase in the product concentration versus the controls. However, after a continuous daily increase in the FSH

concentration in the first 8 days at both CO₂ concentrations, no increase in the concentration was observed in the last day of the process (Figure 12).

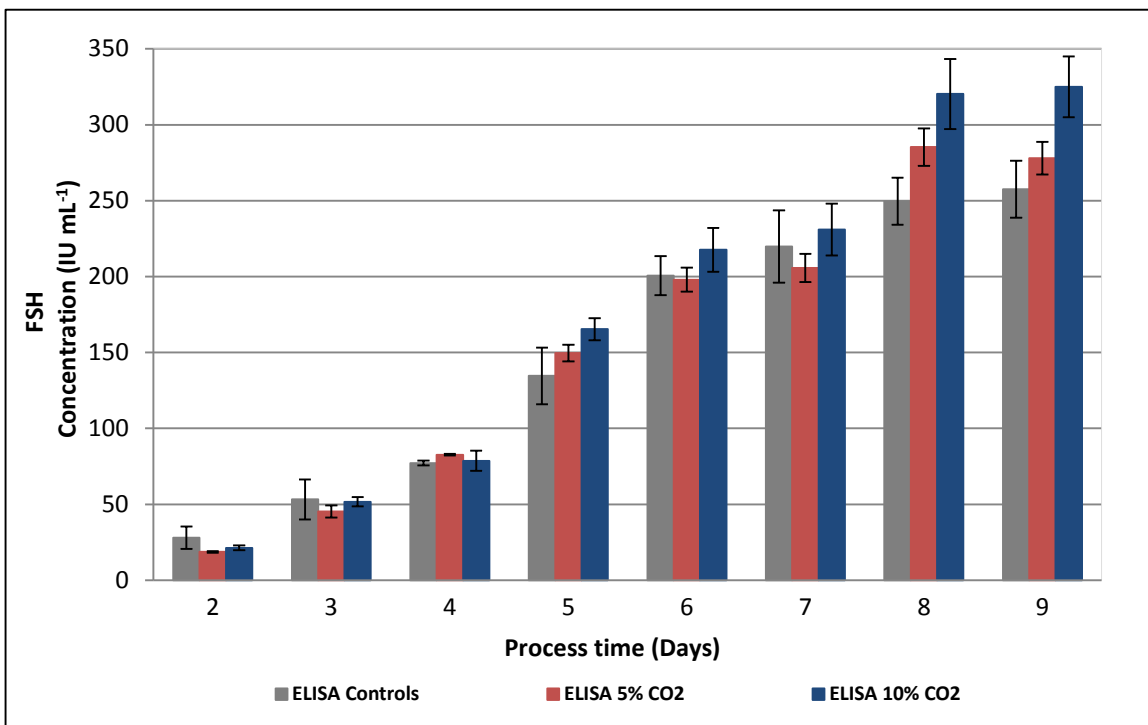


Figure 12: Average FSH concentration measured by ELISA on the DASGIP 750mL bioreactor different CO₂ concentrations. At normal working conditions (controls) n=6, at a 5% CO₂ containing sparged air, n=3, and at 10% CO₂ containing sparged air, n=3.

The cultivation at both a 10% CO₂ containing sparged air and a lower cell density than the control showed a gradual increase in the average product concentration till the last day of the process. It yielded an average FSH concentration on the day of harvest of 345 ± 19 IU/mL. This is 6% higher than the cultivation at 10% CO₂ with normal operating cell density, 12% higher than cultivation at lower viable cell density while sparging normal air, and 34% significantly higher than the controls (P-value <0.05) (Figure 13). Also, this concentration represented 85% of the average concentration produced in the HyClone 50L bioreactor on the last day of cultivation with no significant difference in between (Figure 14).

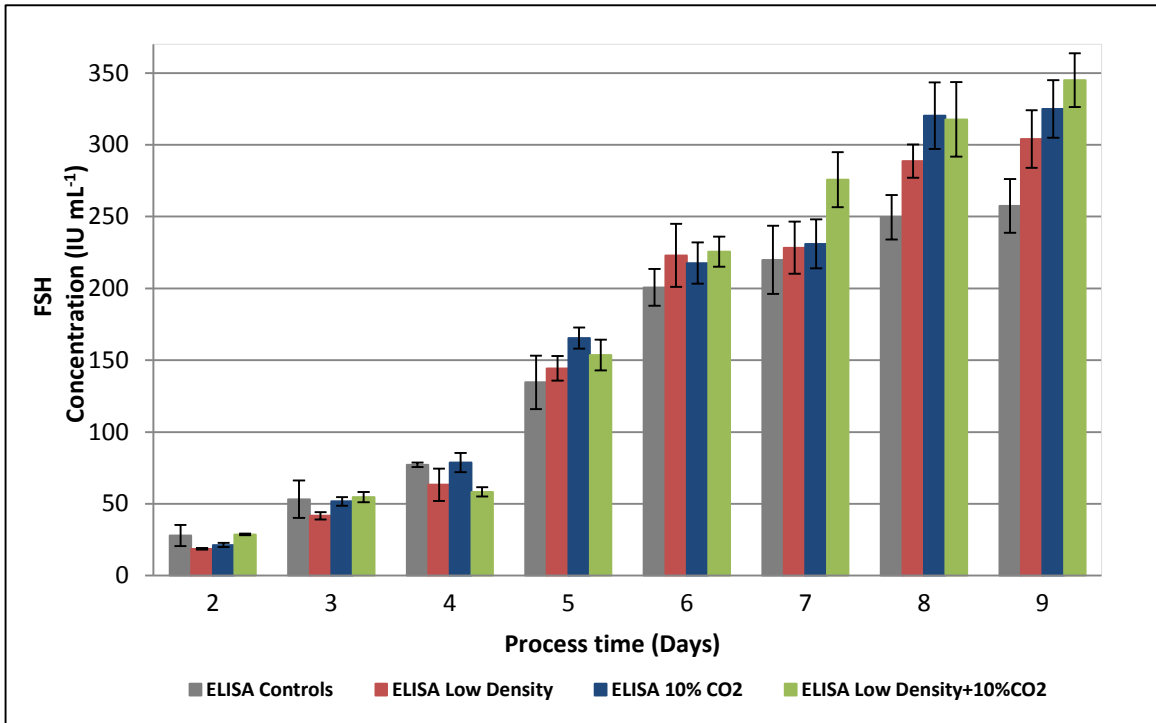


Figure 13: Average FSH concentration measured by ELISA on the DASGIP 750mL bioreactor, at in-house established operating conditions (controls) n=6, at a lower inoculation cell density than the controls, n=3, at 10% CO₂ containing sparged air, n=3, and at both conditions combined (Low starting cell density and 10% CO₂ containing sparged air), n=3.

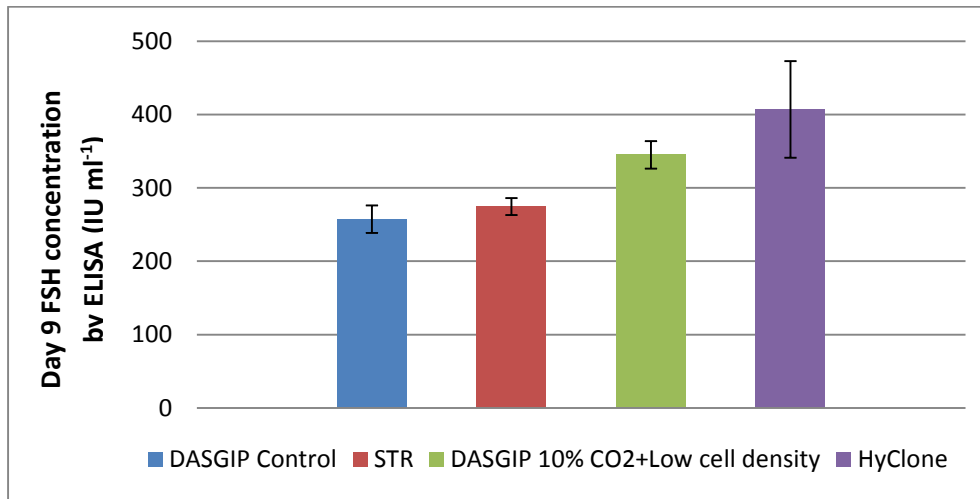


Figure 14: Average FSH concentration measured by ELISA on the day of harvest at the DASGIP bioreactor controls, n=6, the BIOSTAT STR 200L bioreactor operated at 1X and 10X P/V values, n=3, the DASGIP bioreactor at 10% CO₂ containing sparged air and low inoculation cell density, n=3, and at HyClone 50L SUB bioreactor at its normal working conditions, n=3.

Increasing the P/V of the DASGIP 750mL process by ten folds produced an average FSH concentration of 249 ± 36 IU/mL on day 9 with no remarkable difference from the controls throughout the whole process. Only in the first few days the operation at high P/V produced slightly higher amounts of protein (Figure 15).

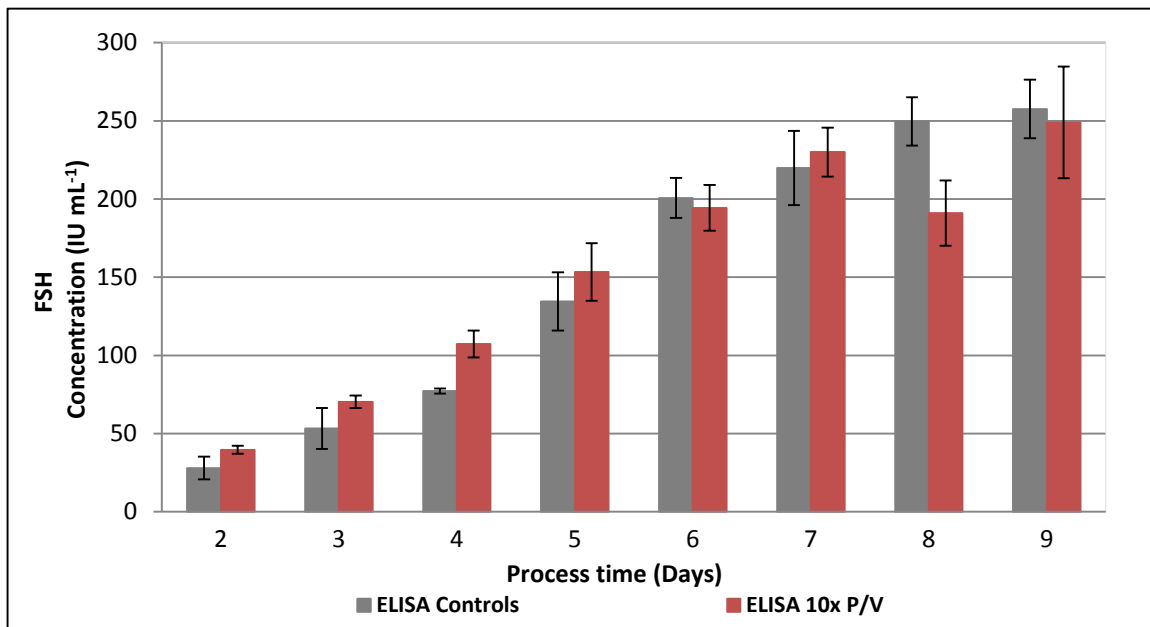


Figure 15: Average FSH concentration measured by ELISA for DASGIP 750mL batches performed at the in-house established operating conditions (Controls), n=6, and at P/V ten folds higher, n=3.

3.6 FSH quantification via HPLC

The HPLC analysis on the day of harvest showed an average of 36.6 ± 3.34 μ g/mL in the HyClone 50L SUB. This represented about 128% of the average product concentration from the two BIOSTAT STR 200L batches performed at ten times higher P/V than that of the HyClone SUB. The third STR batch with adjusted P/V equivalent to that of the HyClone produced 26.8 μ g/mL, which was only 6% less than the average concentration of the other two BIOSTAT STR batches (Figure 16). Comparing the results between the three HyClone 50L SUB runs and the three BIOSTAT STR 200L runs revealed a significant difference with P-value <0.001.

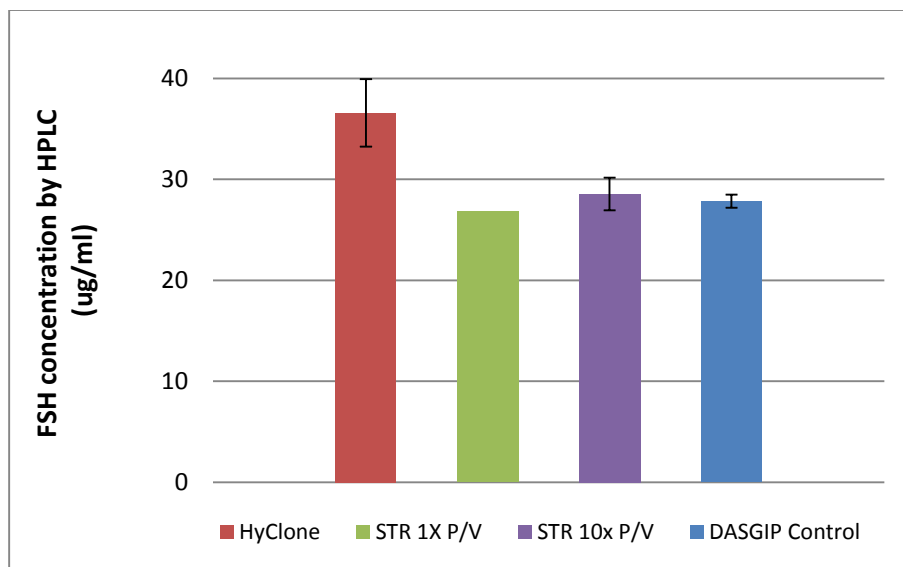


Figure 16: Average FSH concentration measured by HPLC on day of harvest for HyClone 50L, n=3, BIOSTAT STR 200L operated at P/V ten folds greater than that of the HyClone bioreactor, n=2, at P/V equivalent to that of the HyClone, n=1, and DASGIP controls, n=6.

For the DASGIP 750mL bioreactor, the controls produced an average of 27.8 ± 0.64 $\mu\text{g}/\text{mL}$, which is highly comparable to the BIOSTAT STR 200L batches with no significant difference in between, while it represented a significant decrease to only 76% of the average concentration from the HyClone 50L SUB batches (P-value < 0.001).

Higher and lower inoculation densities affected the product concentration inversely. The higher inoculation density yielded an average product concentration of 25.2 ± 0.75 $\mu\text{g}/\text{mL}$, while the lower viable cell density yielded an average of 31.86 ± 0.75 $\mu\text{g}/\text{mL}$ which represented a 15% increase from the controls (Figure 17).

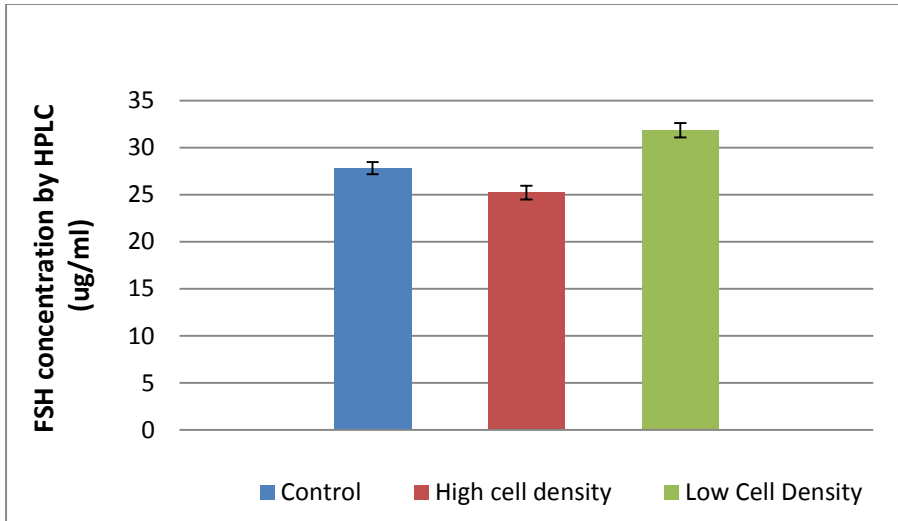


Figure 17: Average FSH concentration measured by HPLC on the day of harvest for the DASGIP 750mL bioreactor at different starting cell densities. At normal working conditions (controls) n=6, at higher inoculation VCD, n=3, and at lower inoculation VCD, n=3.

Increasing the percent of CO₂ in the air introduced into the cell culture at the DASGIP 750mL bioreactor resulted in an average concentration of 29.8 ±0.9 µg/mL with 5% CO₂, and a further increase to 34.46 ±0.66 µg/mL with increasing the CO₂ percentages to 10%. This represented a 124% of the average concentration of the controls (Figure 18), and only 6% less than the average concentration of FSH from the HyClone 50L SUB batches.

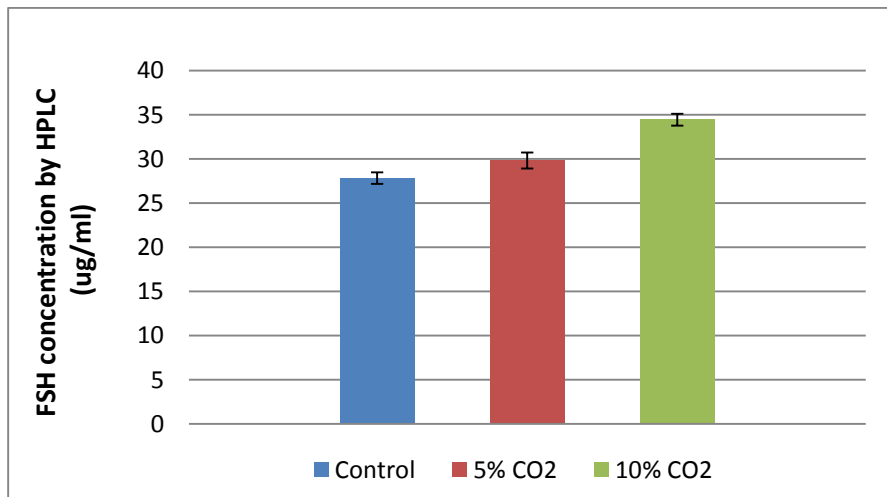


Figure 18: Average FSH concentration measured by HPLC on the day of harvest for the DASGIP 750mL bioreactor at different CO₂ concentrations. At in-house established working conditions (controls) n=6, at 5% CO₂ containing sparged air, n=3 and at 10% CO₂ containing sparged air, n=3.

Cultivation at both 10% CO₂ containing air and a lower cell density than the normal operating conditions yielded an average product concentration of 33.13 ±2.25 µg/mL. This result was comparable to the results from the batches where each parameter was tested separately (Figure 19). This was significantly different from the results of both the DASGIP controls and the BIOSTAT STR 200L runs with P-value <0.05, but not from the HyClone 50L SUB results.

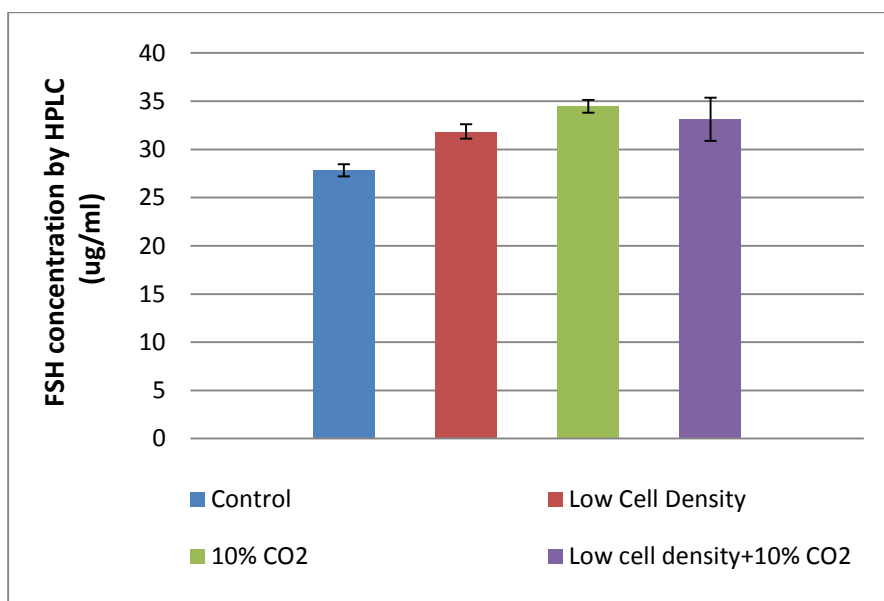


Figure 19: Average FSH concentration measured by HPLC on day 9 of the cultivation process for the DASGIP 750mL bioreactor, at in-house established operating conditions (controls) n=6, at a lower inoculation cell density than the controls (Low Cell Density), n=3, at 10% CO₂ containing sparged air, n=3, and at both conditions combined (Low starting cell density and 10% CO₂ containing sparged air), n=3.

Operating the DASGIP bioreactor on P/V, which is ten-fold higher than its normal operating conditions yielded an average FSH concentration of 28.40 ±2.48 µg/mL, which showed no remarkable difference from the average results of the controls which measured 27.8 ±0.64 µg/mL.

3.7 Cell specific productivity

The average cell specific productivity calculated for the three HyClone 50L SUB batches was $1.61 \times 10^{-6} \pm 0.44 \mu\text{g}/\text{cell}/\text{day}$. The two BIOSTAT STR 200L batches operated at a high P/V which was approximately ten times that of the HyClone 50L SUB bioreactor resulted in cell specific productivities of $0.81 \times 10^{-6} \mu\text{g}/\text{cell}/\text{day}$ and $0.78 \times 10^{-6} \mu\text{g}/\text{cell}/\text{day}$ which were nearly half the average cell specific productivity in the HyClone 50L SUB batches. The third BIOSTAT STR 200L batch operated at low P/V equivalent to that of the HyClone 50L SUB showed no effect on the cell specific productivity, which was calculated to be $0.82 \times 10^{-6} \mu\text{g}/\text{cell}/\text{day}$ (Figure 20). Comparing the results between the three HyClone 50L SUB runs and the three STR runs revealed a significant difference with P-value <0.01 .

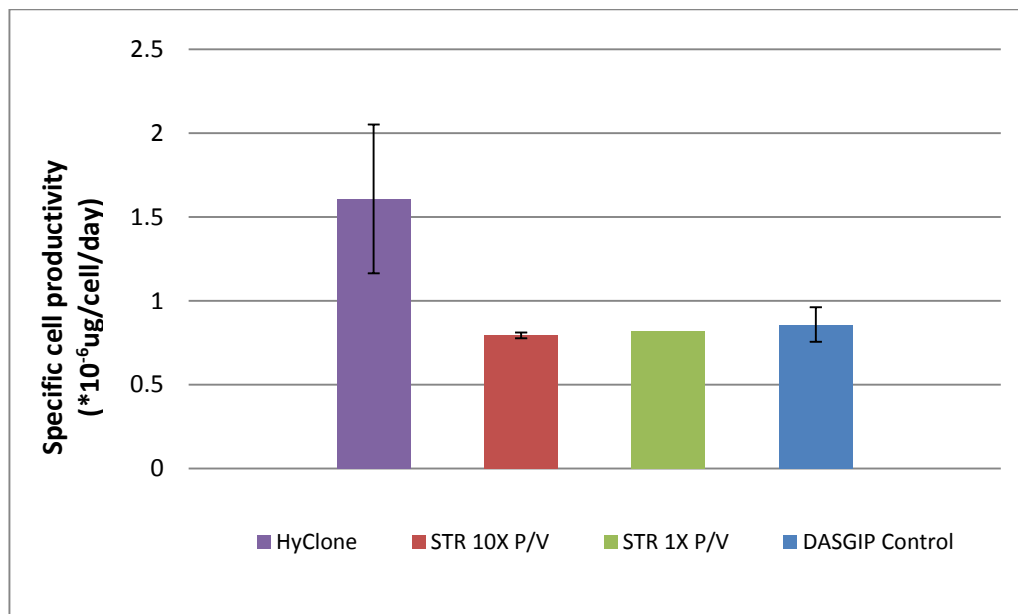


Figure 20: Average cell specific productivity for HyClone 50L, n=3, BIOSTAT STR 200L operated at P/V ten folds greater than that of the HyClone bioreactor, n=2, at P/V equivalent to that of the HyClone, n=1, and DASGIP controls, n=6.

The DASGIP 750mL experiments performed at the in-house established operating conditions showed an average productivity of $0.86 \times 10^{-6} \pm 0.10 \mu\text{g}/\text{cell}/\text{day}$, which was highly comparable with no significant difference from the productivity of the cells observed in the BIOSTAT STR 200L bioreactor batches, and still representing significantly only 53% of the average cell specific productivity in the HyClone 50L SUB (P-value <0.01).

Operating the DASGIP bioreactor at higher cell density resulted in a further decline in the cell productivity. Only $0.55 \times 10^{-6} \pm 0.06 \mu\text{g}/\text{cell}/\text{day}$ were observed. This represented 64% of the cell productivity in the controls. On the other hand, the DASGIP vessels operated with a lower cell density than the controls showed an average productivity of $1.46 \times 10^{-6} \pm 0.07 \mu\text{g}/\text{cell}/\text{day}$. This sharp increase in productivity represented a 70% increase in the productivity from the controls (Figure 21), and about 91% of the average cell productivity in the HyClone 50L SUB bioreactor.

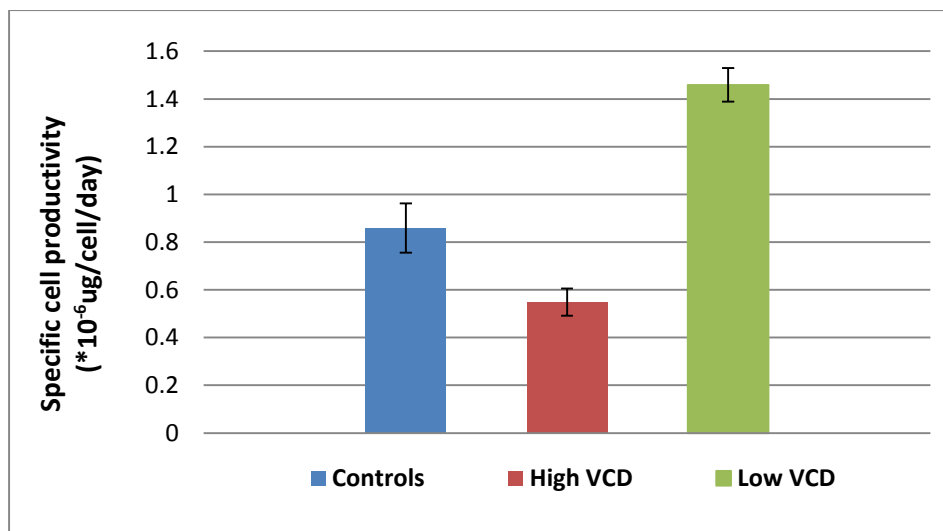


Figure 21: Average cell specific productivity calculated for the DASGIP 750mL bioreactor at different starting cell densities. At in-house established working conditions (controls) n=6, at higher inoculation VCD, n=3, and at lower inoculation VCD, n=3.

Although increasing the percentage of CO_2 in the air sparged in to the culture to 5% showed only a very slight increase in the cell productivity versus the controls, a further increase of the CO_2 percentage to 10% raised the cell productivity by 38%. The averages of the results for the 5% CO_2 were $0.91 \times 10^{-6} \pm 0.04 \mu\text{g}/\text{cell}/\text{day}$ and $1.19 \times 10^{-6} \pm 0.12 \mu\text{g}/\text{cell}/\text{day}$ for the 10% CO_2 (Figure 22).

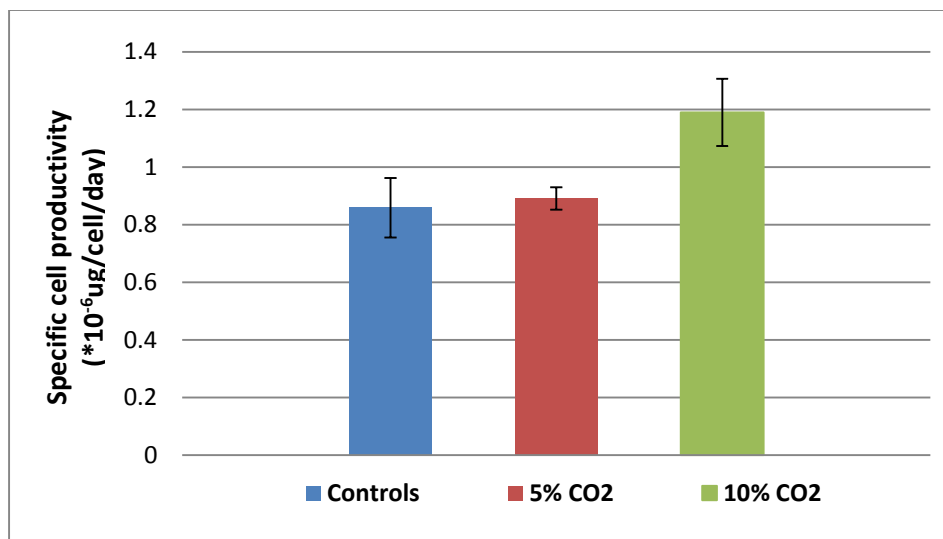


Figure 22: Average cell specific productivity on the DASGIP 750mL bioreactor at different CO₂ concentrations. At normal working conditions (controls) n=6, at 5% CO₂ containing sparged air, n=3 and at 10% CO₂ containing sparged air, n=3.

Combining both parameters that sharply increased the cell productivity (low cell density and 10% CO₂) in the same run led to an average specific cell productivity $1.69 \times 10^{-6} \pm 0.10$ µg/cell/day, which was significantly almost double that of the DASGIP controls (P-value <0.001), 42% higher than the average of the batches cultivated at 10% CO₂ alone, and 16% higher than the average of those cultivated at lower inoculation cell density alone (Figure 23). This was also significantly nearly double the specific productivity from the BIOSTAT STR 200L batches (P-value <0.01), and is even slightly higher than the average productivity in the HyClone 50L SUB batches with no significant difference (Figure 24).

Operating the DASGIP vessels at a P/V which is ten times its normal working parameters' P/V led to a cell specific productivity of $0.78 \times 10^{-6} \pm 0.13$ µg/cell/day, which was very close to the productivities observed in the DASGIP controls as well as the BIOSTAT STR 200L at its both operating parameters.

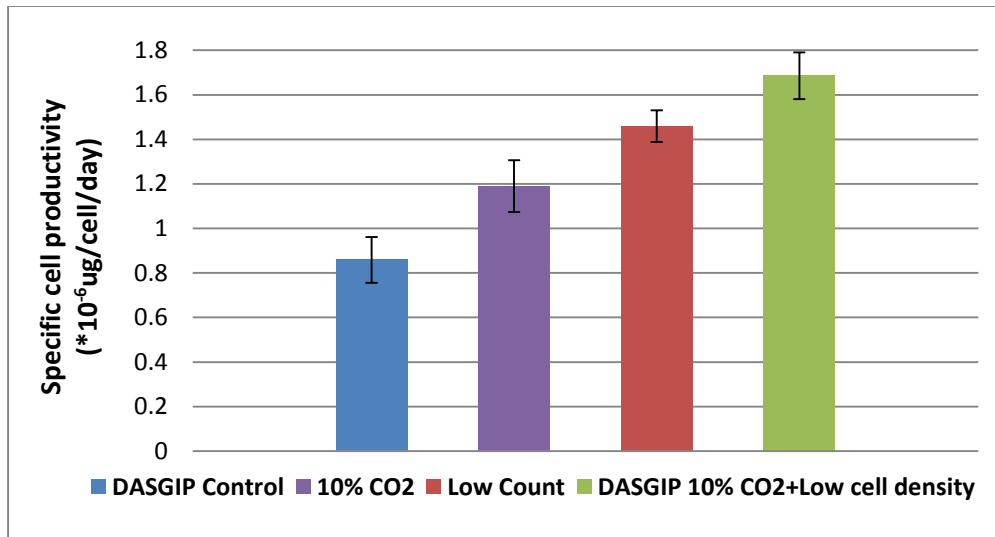


Figure 23: Average specific cell productivity at in-house established operating conditions (controls) n=6, at a lower inoculation cell density than the controls (Low Count), n=3, at 10% CO₂ containing sparged air, n=3, and at both conditions combined (Low starting cell density and 10% CO₂ containing sparged air), n=3.

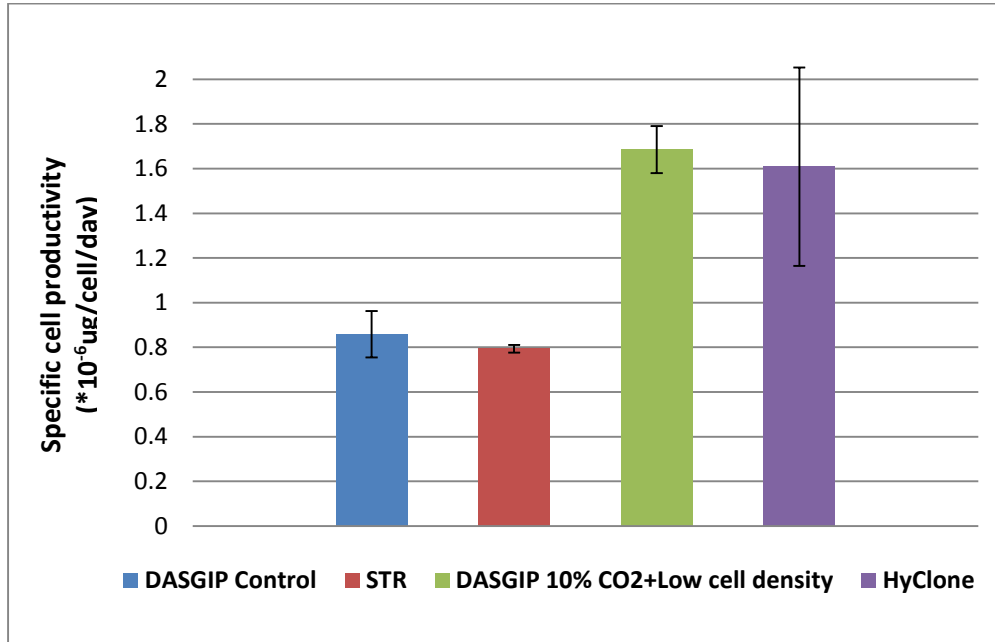


Figure 24: Average cell specific productivity for the DASGIP 750mL bioreactor, at in-house established operating conditions (controls) n=6, at both low cell density and 10% CO₂ containing sparged air, n=3, for the HyClone 50L SUB, n=3, and for the BIOSTAT STR bioreactor, n=3.

3.8 pCO₂ level analysis

The pCO₂ level analysis in the culture showed a comparable level on the production phase between the BIOSTAT STR 200L and the DASGIP controls on one side, the HyClone 50L SUB and the DASGIP operating at 10% CO₂ containing air on the other side.

During the growth phase of the process, the average pCO₂ in the DASGIP controls was between 40 and 50 mmHg, while in the BIOSTAT STR between 70 and 80 mmHg. However, this difference did not last for long. Since in the production phase the pCO₂ levels became highly comparable in the range between 25 and 40 mmHg.

On the other hand, for the HyClone 50L SUB and the DASGIP experiments operated at 10% CO₂, the levels were varying between 80 and 110 mmHg in the growth phase. On the production phase, the levels were decreasing gradually starting from about 90 mmHg on day 4 of the cultivation process till reaching about 60 mmHg on the day of harvest (Figure 25).

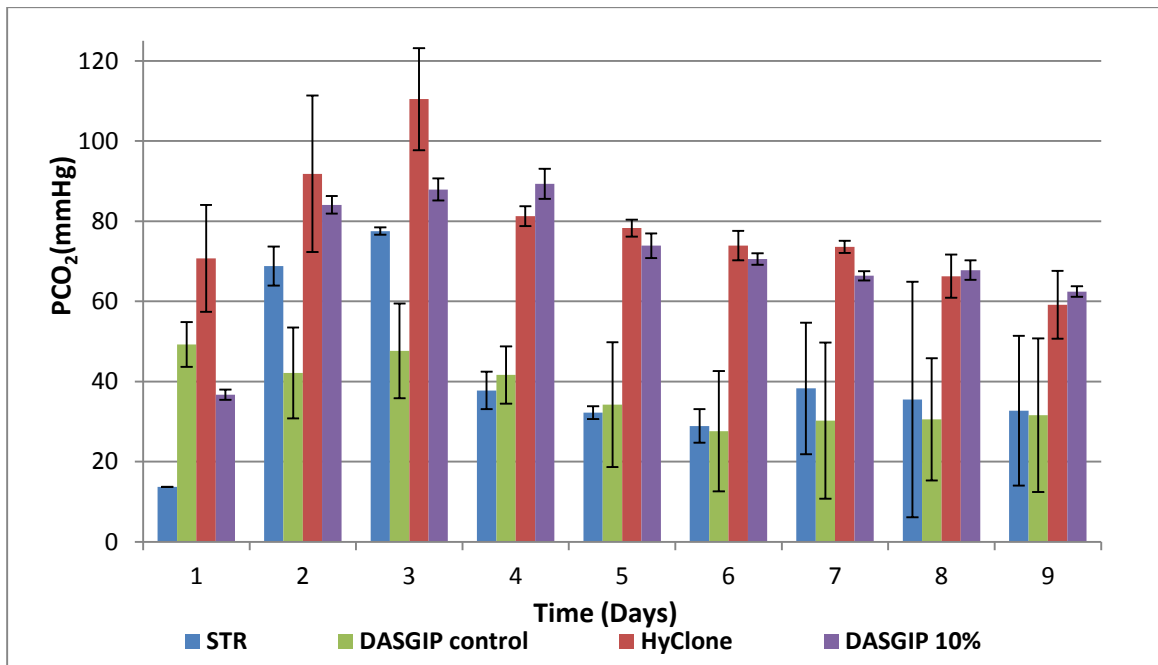


Figure 25: Average pCO₂ level in the BIOSTAT STR, n=3, DASGIP controls, n=6, HyClone SUB, and DASGIP operated at 10% CO₂ containing sparged air, n=6.

3.9 Osmolality level analysis

For osmolality, the levels were highly comparable in the BIOSTAT STR 200L and the DASGIP controls throughout the whole run. The osmolality level increased gradually during the growth phase till it reached 380-400 mOsm/kg on day 3. During the production phase the level was always between 390 and 410 mOsm/kg.

For the HyClone 50L SUB and the DASGIP experiments operated at 10% CO₂ containing sparged air, the highest level in the production phase was between 410 and 425 mOsm/kg on day 3 being slightly higher in the HyClone 50L SUB, while on the production phase the level was in the range of 420-460 mOsm/kg with about 10-20 mOsm/kg higher levels in the DASGIP runs (Figure 26).

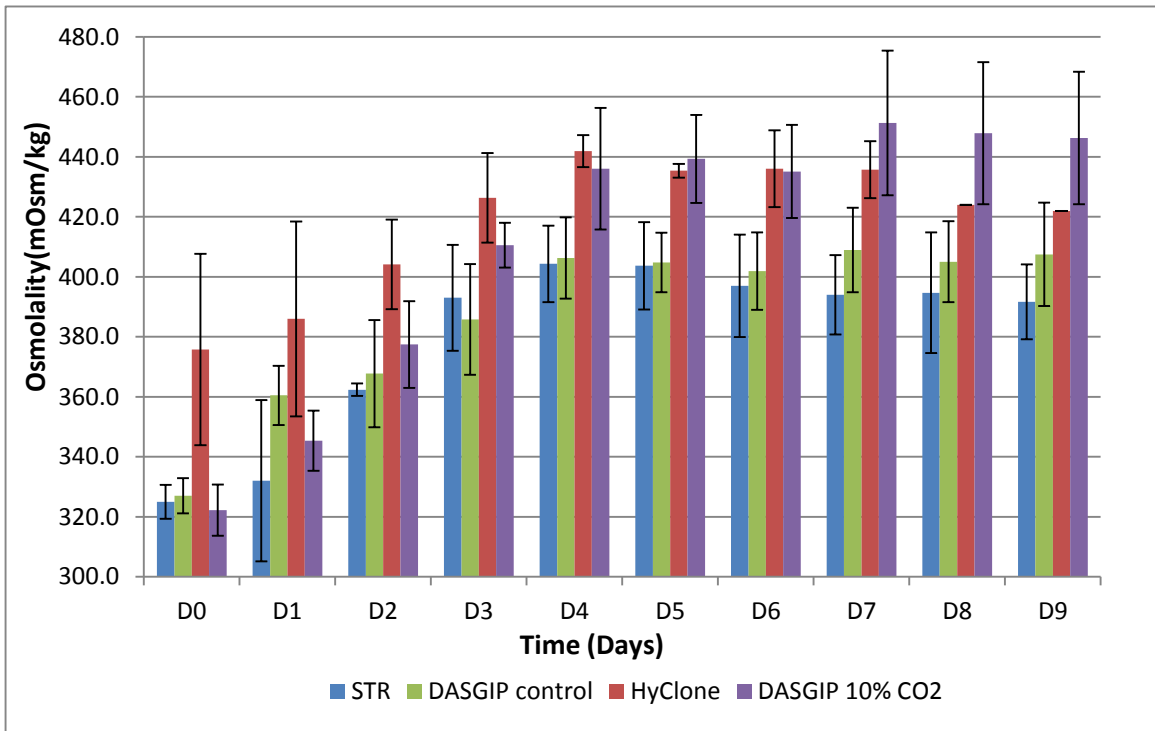


Figure 26: Average Osmolality in the BIOSTAT STR (n=3), DASGIP controls (n=6), HyClone SUB, and DASGIP operated at 10% CO₂ containing sparged air (n=6).

Chapter 4: Discussion

Bioreactors do not operate identically at different scales, and it is not possible to scale up a process based on a single engineering parameter because other parameters will change accordingly. Thus, the most important thing is to identify the critical process parameters and to set up the scale up strategy based on those identified critical parameters.

Taking the scaling up of the production process of rh-FSH from HyClone 50L SUB to the BIOSTAT STR 200L bioreactor as an example, the process was first operated on the 200L bioreactor at its in-house established operating conditions. A big difference was observed in the achieved cell density, cell viability, product concentration as well as the cell specific productivity when compared to those observed in the 50L process. Different cultivation and engineering parameters were tested if they represent potential critical parameters for a successful scale up process and the results are discussed herein.

4.1 Effect of common engineering scale up parameters

The most used criteria for scale up are based on the empirical relationships that correlate P/V and k_{LA} (Vilaca et al., 2000). This relationship accounts for agitation and aeration parameters, which directly influence gas-liquid mass transfer (Yawalkar et al., 2002).

In this study, the calculations revealed that at the in-house established operating conditions, the BIOSTAT STR 200L bioreactor operated at approximately 10 times P/V and 3 times k_{LA} higher than the HyClone 50L SUB. The high value of P/V reaching 78.1 W/m³ was considered as a probable reason for the higher death rate and hence the lower protein concentration produced from the BIOSTAT STR 200L bioreactor (Langheinrich et al., 1998; Junker, 2004). This consideration was due to the fact that P/V in animal cell culture processes is kept at much lower values than that in microbial cultures because of the higher fragility of the mammalian and insect cells. A global average of 10 W/m³ and a range of 1–50 W/m³ were suggested in animal cell culture (Rathore et al., 2008).

Operating the BIOSTAT STR 200L at lower agitation speeds that account for a P/V equivalent to that of the HyClone 50L SUB bioreactor and consequently a k_{LA} about 10-

18% higher than that of the HyClone50L SUB did not result in higher productivity nor better viabilities. Such results are also confirmed by lab scale experiments operated at a 10 folds difference in P/V. Thus, the study was directed to search other factors that might be the possible causes of the different behavior in cell growth, cell death and the protein production.

4.2 Effect of cell density

The main objective of fermentation in industry is to increase productivity (Lee, 1996; Riesenbergr & Guthke, 1999). Productivity is a function of cell density and the amount of product formed by each cell per unit time, what is called the specific cell productivity. Thus, increasing the productivity requires increasing the cell density in the culture as well as the specific productivity for the cell. Cultivation at high cell densities can be a powerful tool for production of recombinant proteins. A recent study has reached a cell density of 1.5-1.8 x10⁷ cells/mL in fed batch culture and 1.27x10⁸ cells/mL for a perfusion culture in a wave bioreactor. Both showed an average specific cell productivity of MAb between 9 and 13 pg/cell/day (Clincke et al, 2013).

However, the results of this study showed an inverse relation between the cell density in culture and the overall productivity. This was attributed to the sharp decline in specific cell productivity with increased cell culture density. It was also observed in experiments of this study that the higher the cell density the faster is the cell death and the lower cell viability at the end of the cultivation. This negative effect of high cell density on the productivity was confirmed by the results from the DASGIP 750mL bioreactor experiments.

The main problems that could arise from high density cell culture include limited availability of DO at high cell densities, accumulation of carbon dioxide to the levels that can decrease growth rates, reduced mixing efficiencies in the fermentor (Lee, 1996), limitation and/or inhibition of substrates essential for growth and accumulation of metabolic by-products to a growth-inhibitory level (Riesenbergr & Guthke, 1999).

Concerning the above-mentioned potential reasons for decreased productivity at high cell densities, substrate inhibition or limitation as well as formation of growth inhibitory byproducts are difficult to discover. This is due to the complexity of the used culture media and the inability to monitor the concentration of every single nutrient or byproduct. However, the levels of glucose and glutamine, being agreed as the main carbon and energy sources in cell culture (Altamirano et al., 2000), together with their byproducts, lactate and ammonia, were monitored throughout the cultivation process. No remarkable differences in glucose, glutamine, lactate and ammonia levels were observed between the BIOSTAT STR 200L and the HyClone 50L SUB bioreactors (Data not shown).

Reduced mixing efficiency was refuted by the fact that the Reynolds number at all operating conditions for both the HyClone 50L SUB and the BIOSTAT STR 200L bioreactors were above 10000, which represents the threshold of turbulent flow (Armenante & Chang, 1998).

Interestingly, Kou et al. described a study in which the specific productivity of recombinant Tumor Necrosis Factor Receptor:Fc Fusion Protein (TNFR-Fc) in CHO cells at cell density of 6×10^6 cells/mL was 68% less than that at cell density 2×10^6 cells/mL. It was revealed that the mRNA level of the recombinant protein at the lower cell density culture was almost twice the level than in the higher cell density culture. It was also proved that the rate of protein assembly and transport from the endoplasmic reticulum to the Golgi were significantly lower at the high cell density culture. Thus, the reduced productivity of the recombinant protein at the high cell density was shown to be correlated with the reduction in both mRNA level and post-translational processing rate (Kou et al., 2011).

4.3 pCO₂ and osmolality

pCO₂ and osmolality are highly correlated in cultures where pH is maintained by base addition. Base addition at elevated pCO₂ increases bicarbonate concentration and hence increases osmolality. At equilibrium, [CO_{2(aq)}], pH, and [HCO₃⁻] are related by the Henderson-Hasselbach equation for CO₂ dissociation:

$$\text{pH} = \text{pK} + \log\left(\frac{[\text{HCO}_3^-]}{[\text{CO}_{2(\text{aq})}]}\right) \quad \text{Equation 8}$$

The [CO_{2(aq)}] is directly proportional to pCO₂ at equilibrium. Therefore, the bicarbonate concentration, and hence the osmolality, will be proportional to pCO₂ at constant pH (Kimura & Miller, 1996). Typically, a cell culture medium is designed to have an osmolality in the range of 260-320 mOsm/kg, to mimic the osmolality of serum (290mOsm/kg) (Ozturk & Palsson, 1991).

In the current study, the aim was to examine if the difference in cell growth rate and product titer between the HyClone 50L SUB and the BIOSTAT STR 200L bioreactors can be correlated to the difference in the pCO₂ and osmolality observed throughout the process performed in the two bioreactors. This was tested on the lab scale DASGIP bioreactor where comparable pCO₂ and osmolality values between the DASGIP controls and the BIOSTAT STR 200L bioreactor yielded a comparable growth rate and product titer. While on the other hand, the higher pCO₂ and osmolality in the DASGIP units with 10% CO₂ containing sparged air yielded also a comparable growth rate and product titer to the HyClone 50L SUB. This suggests that one reason for the difference in the rate of cell growth and the protein production between the HyClone 50L and the BIOSTAT STR 200L bioreactor may be the difference in the culture pCO₂ and osmolality. The pCO₂ in the 10% CO₂ DASGIP experiments was about 90 mmHg on the growth phase and 60-80 on the production phase compared to 50 mmHg and 30-40 mmHg for the controls. While the osmolality was 400-410 mOsm/kg at the growth phase and 430-445 mOsm/kg at the production phase compared to 390 mOsm/kg and 400-410 mOsm/kg for the controls. Such small differences of only 10 or 20 units resulted in differences as big as 26% increase in the product titer on ELISA, 24% on HPLC, a decrease by 0.65 million viable cells/mL in

the maximum viable cell density and around 10% increase in viability at the end of the process. The results of reduced cell growth rate and increased productivity match the findings from the previous literature (Kurano et al., 1990; Kimura & Miller, 1996; Oyaas et al., 1994; Lee & Park, 1995) where high levels of osmolality as well as pCO₂ were found to be detrimental to mammalian cell culture in terms of cell growth rate and productivity.

One study showed that the specific growth rate in hybridoma culture was reduced by about 50% when osmolality increased from 360 to 450 mOsm/kg (Kurano et al., 1990). Another study showed that a combination of elevated pCO₂ and osmolality reduced specific growth rate up to 45%, while it had no effect or even increased productivity of tissue plasminogen activator (tPA) producing CHO cells (Kimura & Miller, 1996). A third study reported a decrease in cell growth and an increase in antibody production from hybridoma cells at high osmolarities (Oyaas et al., 1994). And a fourth study showed that for CHO cells producing antibody fusion protein B1, the combined effect from high pCO₂ (140-160 mmHg) and elevated osmolality (400-450 mOsm/kg), as compared to elevated osmolality alone, caused more decrease in viable cell density, and it has also increased the specific cell productivity (Zhu et al., 2005). It was also reported that the magnitude of the increase of antibody productivity from hyperosmotic stress is cell line dependent (Lee & Park, 1995).

However, the observation of enhanced viability at the end of the process at high osmolality and pCO₂ from the current study is conflicting to the reported negative effect of elevated pCO₂ and osmolality on the cell viability. Thus, it can be concluded that the high cell density was the major cause of the reduced viabilities of the culture, and this effect is achieved through another mechanism rather than the accumulation of CO₂ as a waste product in the culture medium.

Although many studies reported the relation between elevated pCO₂ and/or osmolality on the growth and the productivity behaviors of mammalian cell culture, none of them identified the real mechanism of such an effect. Some studies declared that the high pCO₂ levels reduce intracellular pH (pH_i), even when the medium pH is controlled (Krapf et al.,

1988). This was attributed to the fact that CO₂ can readily diffuse across the cell membrane, where it can hydrate and then dissociate into H⁺ and HCO₃⁻ (Alberts et al., 1989). These pH_i changes can affect the activity of cytosolic enzymes, it can alter the cell metabolism (Madhus, 1988), and it can also change the pH in the endoplasmic reticulum and/or the Golgi apparatus which could alter protein processing and secretion (Thorens & Vassalli, 1986).

A very interesting finding was reported for the addition of sodium propionate to CHO cell culture which resulted in suppression in the cell growth rate, but enhanced the FSH production (Yoon & Ahn, 2007). Propionic acid is a weak acid, which is membrane permeable. Exposing animal cells to propionic acid as well as other weak organic acids like butyric or benzoic acid has been a classical approach to reduce the intracellular pH (Roos & Boron, 1981). The enhanced production of rh-FSH from CHO cells with both propionate addition and elevated pCO₂ suggests that lowering the intracellular pH is a probable cause of such an effect.

The reason for the difference in pCO₂ levels between the HyClone 50L SUB and the BIOSTAT STR 200L is assumed to be correlated to their different sparger types and pore size. HyClone 50L SUB has a porous frit gas microsparger with pores size of 25 μm (Thermo Scientific HyClone Single-Use Bioreactor (S.U.B.) 50 L datasheet, website: <https://fscimage.thermoscientific.com/images/D17255~.pdf>), while the BIOSTAT STR 200L bioreactor incorporates a classical ring sparger with 0.8mm holes (Noack et al., 2010b). Microspargers significantly increase the gas-medium interfaces and hence provide higher O₂ mass-transfer coefficients (Fenge & Lüllau, 2006). Further, microspargers have a lower efficiency in CO₂ removal. This was attributed to the fact that the smaller bubbles are more easily saturated with CO₂ than the larger ones (Czermak et al., 2009).

Chapter 5: Conclusions and Future Recommendations

5.1 Conclusions

Experiments on the lab scale DASGIP bioreactor revealed that the critical parameters in the upstream process for production of rh-FSH from CHO cells were the cell density and pCO₂ and/or osmolality levels in the culture. These parameters can be considered as the major potential factors causing the differences in the maximum cell density, cell viability, product formation and cell productivity between the HyClone 50L SUB and the BIOSTAT STR 200L bioreactors.

The high cell density was shown to reduce the specific cell productivity as well as the total product concentration. The higher pCO₂ and osmolality levels in the culture were shown to increase the specific cell productivity and the total product concentration. They were also shown to exert a slight reduction in the cell growth rate.

Commonly used scale up parameters like the P/V and k_{LA} are not critical factors to be kept constant during the scaling of this process at least at the ranges tested in this study.

5.2 Future Recommendations

It is recommended to adjust the operating parameters of the BIOSTAT STR 200L bioreactor, by reducing the inoculation cell density, and introducing higher levels of CO₂ in the sparged air in order to achieve pCO₂ levels comparable to those of the HyClone 50L SUB.

It is further recommended to carry out lab scale experiments to study the individual effects of raising pCO₂ and osmolality levels on the process. This can be done by changing one parameter and keeping the other constant in order to identify the exact process critical parameter.

To further assess the reason for reduced productivity at high cell density, experiments can be performed to assess the level of mRNA and the rate of protein processing at different culture cell densities. Also, a detailed nutrients and metabolites analysis can reveal some

essential nutrients or inhibitory byproducts that might exceed their critical limits at high cell densities

In addition it is recommended to test whether the enhanced FSH production is associated with low intracellular pH values by adding different reagents to the culture that alter the intracellular pH.

References

Alam, Z., Muhd, N. H., Razali, F., (2005). Scale-up of stirred and aerated bioengineering bioreactor based on constant mass transfer coefficient. *J. Teknologi.*, 43, 95-110.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., & Watson, J. D. (1989). *Molecular biology of the cell*. 2nd edition. Garland, New York.

Altamirano, C., Paredes, C., Cairo, J. J., & Godia, F. (2000). Improvement of CHO cell culture medium formulation: simultaneous substitution of glucose and glutamine. *Biotechnol. Prog.*, 16, 69-75.

Armenante, P. M. & Chang, G. M. (1998). Power Consumption in Agitated Vessels Provided with Multiple-Disk Turbines. *Industrial & Engineering Chemistry Research*, 37, 284-291.

Bassett, R. M. & Driebergen, R. (2005). Continued improvements in the quality and consistency of follitropinalfa, recombinant human FSH. *Reprod. Biomed. Online*, 10, 169-177.

Bylund, F., Collet, E., Enfors, S. O., & Larsson, G. (1998). Substrate gradient formation in the large-scale bioreactor lowers cell yield and increases by-product formation. *Bioprocess Engineering*, 18, 171-180.

Catapano, G., Czermak, P., Eibl, R., Eibl, D., & Portner, R. (2009). Bioreactor Design and Scale-Up. In *Cell and Tissue Reaction Engineering* (pp. 173-259). Springer Berlin Heidelberg.

Chu, L. & Robinson, D. K. (2001). Industrial choices for protein production by large-scale cell culture. *Curr. Opin. Biotechnol.*, 12, 180-187.

Clincke, M. F., Mölleryd, C., Samani, P. K., Lindskog, E., Fäldt, E., Walsh, K., & Chotteau, V. (2013). Very high density of Chinese hamster ovary cells in perfusion by

alternating tangential flow or tangential flow filtration in WAVE bioreactor™—part II: Applications for antibody production and cryopreservation. *Biotechnology progress*, 29(3), 768-777.

Czermak, P., Portner, R., & Brix, A. (2009). Special Engineering Aspects. In *Cell and Tissue Reaction Engineering* (pp. 83-172). Springer Berlin Heidelberg.

Daya, S. (2002). Updated meta-analysis of recombinant follicle-stimulating hormone (FSH) versus urinary FSH for ovarian stimulation in assisted reproduction. *Fertil. Steril.*, 77, 711-714.

De Wilde, D., Noack, U., Kahlert, W., Barbaroux, M., & Greller, G. (2009). Bridging the gap from reusable to single-use manufacturing with stirred, single-use bioreactors. *BioProcessInt*, 7(4).

Dibiasi, K., Jornitz, M. W., Gottschalk, U., & Priebe, P. M. (2007). Disposable biopharmaceutical processes-myth or reality?. *Biopharm international*, (OCT), 19-24.

Doran, P. M. (1995). 9 - Mass Transfer. In P.M.Doran (Ed.), *Bioprocess Engineering Principles* (pp. 190-217). London: Academic Press.

Eibl, R. & Eibl, D. (2009). Application of disposable bag bioreactors in tissue engineering and for the production of therapeutic agents. *Adv. Biochem. Eng. Biotechnol.*, 112, 183-207.

El Taieb, S., Bauer, I., Rohde, J., & Mueller, F. (2010). Disposable pilot scale process for the production of a CHO derived recombinant follicle stimulating hormone. Poster presented at Biotech 2010, Wädenswill, Switzerland.

Fenge, C., & Lüllau, E. (2006). Cell Culture Bioreactors. In *Cell Culture Technology for Pharmaceutical And Cell-Based Therapies* (pp. 155-224). CRC Press.

Forgione, P. & Van Trier, M. (2006). The end for stainless steel. *BioProcess Int.*, 4, 58-62.

Gallagher, P. M., Hodge, G., Guertin, P., Chew, L., & Deloggio, T. (2011). Single-Use Bioreactor Platform for Microbial Fermentation. *Single-Use Technology in Biopharmaceutical Manufacture*, 241-250

Genetic Engineering and Biotechnology News. (2006). Disposable Bioreactors Gaining Favor 26,1.<<http://www.genengnews.com/articles/chitem.aspx?aid=1807>>.

Holland, I. A. & Chapman, F.S. (1966). *Liquid Mixing and Processing in Stirred Tanks*. Reinhold: New York.

Howles, C. M. (1996). Genetic engineering of human FSH (Gonal-F). *Hum.Reprod.Update.*, 2, 172-191.

Ju, L. K. & Chase, G. G. (1992). Improved scale-up strategies of bioreactors. *Bioprocess Eng.*, 8, 49-53.

Junker, B. H. (2004). Scale-up methodologies for Escherichia coli and yeast fermentation processes. *J.Biosci.Bioeng.*, 97, 347-364.

Kane, J. (2012). Measuring kLa for Better Bioreactor Performance. *BioProcess Int.*, 10,46-49.

Kimura, R. & Miller, W. M. (1996). Effects of elevated pCO₂ and/or osmolality on the growth and recombinant tPA production of CHO cells. *Biotechnology and Bioengineering*, 52, 152-160.

Kou, T. C., Fan, L., Ye, Z. Y., Zhou, Y., Liu, X. P., Zhao, L. et al. (2011). Process analysis of reduced specific productivity of TNFR-Fc in Chinese hamster ovary cells at high cell density. *Process Biochemistry*, 46, 1492-1499.

Krapf, R., Berry, C. A., Alpern, R. J., & Rector, F. C., Jr. (1988). Regulation of cell pH by ambient bicarbonate, carbon dioxide tension, and pH in the rabbit proximal convoluted tubule. *J.Clin.Invest*, 81,381-389.

Kurano, N., Leist, C., Messi, F., Kurano, S., & Fiechter, A. (1990). Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. *J. Biotechnol.*, *15*, 113-128.

Langheinrich, C. & Nienow, A. W. (1999). Control of pH in large-scale, free suspension animal cell bioreactors: alkali addition and pH excursions. *Biotechnology and Bioengineering*, *66*, 171-179.

Langheinrich, C., Nienow, A. W., Eddleston, T., Stevenson, N. C., Emery, A. N., Clayton, T. M. et al. (1998). Liquid Homogenization Studies in Animal Cell Bioreactors of up to 8 m³ in Volume. *Food and Bioproducts Processing*, *76*, 107-116.

Lee, G. & Park, S. (1995). Enhanced specific antibody productivity of hybridomas resulting from hyperosmotic stress is cell line-specific. *Biotechnol Lett*, *17*, 145-150.

Lee, S. Y. (1996). High cell-density culture of Escherichia coli. *Trends Biotechnol.*, *14*, 98-105.

Ma, N., Koelling, K. W., & Chalmers, J. J. (2002). Fabrication and use of a transient contractional flow device to quantify the sensitivity of mammalian and insect cells to hydrodynamic forces. *Biotechnology and Bioengineering*, *80*, 428-437.

Ma, N., Mollet, M., & Chalmers J.J. (2006). Aeration, Mixing and Hydrodynamics in Bioreactors. In *Cell Culture Technology for Pharmaceutical And Cell-Based Therapies* (pp. 225-248). CRC Press.

Madhus, I. H. (1988). Regulation of intracellular pH in eukaryotic cells. *Biochem. J.*, *250*, 1-8.

Marks, D. M. (2003). Equipment design considerations for large scale cell culture. *Cytotechnology*, *42*, 21-33.

Menisher, T., Metghalchi, M., & Gutoff, E. B. (2000). Mixing studies in bioreactors. *Bioprocess Engineering*, *22*, 115-120.

Nienow, A. W. (2006). Reactor engineering in large scale animal cell culture. *Cytotechnology*, 50, 9-33.

Nienow, A. W. (1998). Hydrodynamics of Stirred Bioreactors. *Applied Mechanics Reviews*, 51, 3-32.

Nienow, A.W., (2003). Aeration-biotechnology. In *Kirk Othmerencyclopaedia of chemical technology*, 5thedn. Wiley Interscience, New York.

Noack, U., Brandt, S., De Wilde, D., & Greller, G. (2010a). Evaluation of the BIOSTAT CultiBag STR family in terms of scalability. Poster presented at Biotech 2010, Wädenswil, Switzerland.

Noack, U., De Wilde, D., Verhoeve, F., Balbirnie, E., Kahlert, W., Adams, T. et al. (2010b). Single-Use Stirred Tank Reactor BIOSTAT CultiBag STR: Characterization and Applications. In *Single-Use Technology in Biopharmaceutical Manufacture* (pp. 225-240). John Wiley & Sons, Inc.

Oh, S. K. W., Nienow, A. W., Al-Rubeai, M., & Emery, A. N. (1989). The effects of agitation intensity with and without continuous sparging on the growth and antibody production of hybridomacells. *Journal of Biotechnology*, 12, 45-61.

Oyaas, K., Ellingsen, T. E., Dyrset, N., & Levine, D. W. (1994). Hyperosmotic hbridoma cell cultures: Increased monoclonal antibody production with addition of glycine betaine. *Biotechnology and Bioengineering*, 44, 991-998.

Ozturk, S. S. & Palsson, B. O. (1991). Growth, metabolic, and antibody production kinetics of hybridoma cell culture: 1. Analysis of data from controlled batch reactors. *Biotechnol.Prog.*, 7, 471-480.

Rathore, A. S., Green, K., Hashimura, Y., & Nyberg, G. (2008). Modeling of Biopharmaceutical Processes—Part 1: Microbial and Mammalian Unit Operations. *BioPharm International*, 21(6).

Riesenberg, D. & Guthke, R. (1999). High-cell-density cultivation of microorganisms. *Appl. Microbiol. Biotechnol.*, 51, 422-430.

Roos, A., & Boron, W. F. (1981). Intracellular pH. *Physiological Reviews*, 61, 296-434.

Schmidt, F. R. (2005). Optimization and scale up of industrial fermentation processes. *Appl. Microbiol. Biotechnol.*, 68, 425-435.

Seamans, T. C., Fries, S., Beck, A., Wurch, T., Chenu, S., Chan, C., ... & Chartrain, M. (2008). Cell Cultivation Process Transfer and Scale-Up. *BioProcess International*.

Selker, M. & Paldus, B. (11-6-2008). Disposable bioreactor system. Google Patents. Ref Type: Generic

Shukla, A. A., & Gottschalk, U. (2013). Single-use disposable technologies for biopharmaceutical manufacturing. *Trends in biotechnology*, 31(3), 147-154.

Shuler, M. L., & Kargi, F. (2002). *Bioprocess engineering* (pp. 120-133). Upper Saddle River. Upper Saddle River.: Prentice Hall.

Stoker, E. B. (2011). Comparative studies on scale-up methods of single-use bioreactors.

Thorens, B. & Vassalli, P. (1986). Chloroquine and ammonium chloride prevent terminal glycosylation of immunoglobulins in plasma cells without affecting secretion. *Nature*, 321, 618-620.

Van't Riet, K. (1979). Review of Measuring Methods and Results in Nonviscous Gas-Liquid Mass Transfer in Stirred Vessels. *Industrial & Engineering Chemistry Process Design and Development*, 18, 357-364.

Vilaca, P. R., Badino, J., Facciotti, M. C. R., & Schmidell, W. (2000). Determination of power consumption and volumetric oxygen transfer coefficient in bioreactors. *Bioprocess Engineering*, 22, 261-265.

Wise, W. S. (1951). The measurement of the aeration of culture media. *J. Gen. Microbiol.*, 5, 167-177.

Yawalkar, A. A., Heesink, A., Versteeg, G. F., & Pangarkar, V. G. (2002). Gas—Liquid Mass Transfer Coefficient in Stirred Tank Reactors. *The Canadian Journal of Chemical Engineering*, 80(5), 840-848.

Yoon, S. & Ahn, Y. H. (2007). Application of sodium propionate to the suspension culture of Chinese hamster ovary cells for enhanced production of follicle-stimulating hormone. *Biotechnol. Bioprocess Eng.*, 12, 497-501.

Żerek, B., & Różga, P. Recombinant Protein Therapeutics—The future is here.

Zhu, M. M., Goyal, A., Rank, D. L., Gupta, S. K., Vanden, B. T., & Lee, S. S. (2005). Effects of elevated pCO₂ and osmolality on growth of CHO cells and production of antibody-fusion protein B1: a case study. *Biotechnol. Prog.*, 21, 70-77.

Zwart-van Rijkom, J. E. F., Broekmans, F. J., & Leufkens, H. G. M. (2002). From HMG through purified urinary FSH preparations to recombinant FSH: a substitution study. *Human Reproduction*, 17(4), 857-865.