Production and Glycosylation of a Recombinant Protein from Chinese Hamster Ovary (CHO) Cells

By

Ann-Marie de Villiers

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DECLARATION OF OWN WORK

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EXECUTIVE SUMMARY

ENGLISH

Recombinant glycoproteins are important biopharmaceuticals, providing solutions for numerous previously untreatable illnesses, in everything from cancer to infertility. Most recombinant biopharmaceuticals are produced in mammalian cells due to their ability to provide the correct post-translational processing for use in humans. The post-translation processing influences many of the protein's properties including pharmacokinetics, bioactivity, secretion, half-life, solubility, recognition and antigenicity. The aim of this thesis is to further study the upstream production of a glycosylated recombinant protein produced by Chinese hamster ovary (CHO) cells on production scale within the confines of an existing process.

The process in question uses adherent CHO cells to produce a glycosylated recombinant hormone. As with most recombinant protein production processes, this process has two sections to the upstream production: a seed train to grow enough cells to inoculate production, and a production section, which focuses on the production of a recombinant protein. The seed train is predominantly conducted in roller bottles, while the production section takes place in perfusion bioreactors, where the cells are attached to microcarriers, with spin-filters for cell retention. The whole process uses medium with serum.

There are two process challenges regarding an existing recombinant-protein production process:

1. The gradual increase, over the past several campaigns, of the final population doubling level of the cells (which must remain within certain specified limits) at the end of the seed train.
2. The low glycosylation levels of the product seen in certain campaigns, which meant that a certain number of final product batches were below the specified acceptable glycosylation limits.

Following a literature survey several controlled process variables were chosen for investigation and hypotheses made on their effect on the seed train or glycosylation.

To investigate their effect on the PDL and cell growth in the seed train:

- Medium volume: decreasing the medium volume will yield a lower PDL due to slower cell growth caused by lower glucose availability.
- Seeding density: if cells obtain confluence by the time they are harvested, decreasing the seeding density will yield a higher PDL.
- Cultivation temperature: decreasing the temperature ought to decrease the growth rate.
- Medium feed temperature: there will be no significant difference to the cell culture when pre-heated or cold medium is used.

- Aeration: using vent caps will increase the oxygen content of the medium in the roller bottles and the cell growth, yielding a higher PDL.

To investigate their effect on glycosylation during production:

- pH: better glycosylation will be seen at pH 6.9, than at pH 6.7.
- Perfusion rate: a higher perfusion rate will lead to better glycosylation due to increased glucose and glutamine concentrations.

In the seed train, the only factor that significantly influenced the final PDL was the seeding density. Cell growth was inhibited once cells reached confluence, so lowering the seeding density lead to a higher PDL. It is recommended to use a high seeding density to ensure a lower PDL.

Historic data indicated that the seeding density was not the cause of the apparent increase of the final PDL, as all previous campaigns had been seeded with a high seeding density. What then became apparent was that the final PDL remained relatively constant during a campaign and that the increase in final PDL occurred between campaigns. It appears that the apparent increase in the final PDL is due to differences in cell counting between operators as each new campaign was managed by different operators. It is recommended that a mechanical cell counter be used to verify cells counts and to maintain a standard between campaigns.

In the bioreactors, varying the pH proved to have no significant effect on the glycosylation levels. However, both the initial perfusion rate and the specific perfusion rate proved to be important from both historical data and the data generated during these experiments.

Lower levels of the initial perfusion rate lead to better glycosylation and it is recommended that an initial perfusion rate of 1.0 volumes/day be used. The relationship between the specific perfusion rate and the glycosylation appears to be non-linear and requires further study, for now it is recommended that the specific perfusion rate be kept below 0.3 volumes/day/10^9 cells.

Probable reasons for the unsatisfactory glycosylation seen in certain runs could also be proposed from these two factors:

- RP33-133 : Very high specific perfusion rate
- RP32-135 : High initial perfusion rate and very high specific perfusion rate
- RP32-138 : High initial perfusion rate
- RP33-139 : High initial perfusion rate

Further research is recommended into the effect of the specific perfusion rate as well as the specific glucose consumption rate and the specific glutamine concentration on the glycosylation.

AFRIKAANS

Rekombinante glikoproteïene is baie belangrike biofarmaseutiese produkte wat oplossings bied vir talle voorheen ongeneeslike siektes in alles van kanker tot onvrugbaarheid. Meeste rekombinante farmaseutiese produkte word gemaak deur diere-selle as gevolg van hulle bevoegtheid om die korrekte na-translasie stappe te volg sodat die produkte in mense gebruik kan word. Die na-translasie stappe beïnvloed baie van die proteïene se karaktertreke insluitende die farmakokinetika, bioaktiwiteit, uitskeiding, half-leeftyd, oplosbaarheid, herkenbaarheid and antigeniciteit. Die doel van hierdie tesis is om die stroomop produksie van ‘n rekombinante glikoproteïene vervaardig deur Chinese hamster ovariale (CHO) selle verder te bestudeer binne die grense van ‘n bestaande proses op grootskaalse vlak.

Die huidige proses gebruik CHO selle om ‘n rekombinante glikohormoon te produseer. Soos meeste prosesse wat rekombinante proteïene produseer bestaan die stroomop gedeelte van die proses uit twee dele: ‘n saad trein wat genoeg selle maak vir produksie en ‘n produksie gedeelte wat fokus op die vervaardiging van die glikoproteïen. Die saad trein bestaan hoofsaaklik uit roller bottels terwyl produksie plaasvind in perfusie bioreaktors waar die selle op “microcarriers” groei, met spin-filters om die selle binne die bioreaktors te hou; die hele proses gebruik medium met serum.

Daar is twee probleme in die stroomop gedeelte van die bestaande proses:

1. Die geleidelike toename oor die afgelope paar jaar van die finale verdubbelingsvlak van die selle aan die einde van die saad trein

2. Die lae glukosilering van die eindproduk wat veroorsaak dat sekere lotnommers buite spesifikasie is

Na ‘n literatuur studie, was seker beheerde proses parameters gekies om verder te bestudeer en hipotesisse gemaak oor hulle effek op die saad trein of die vlak van glukosilering.

Die volgende faktore is bestudeer vir hulle effek op die finale verdubbelingsvlak van die selle in die saad trein:

- Medium volume: ‘n laer medium volume sal lei tot a laer verdubbelingsvlak van die selle as gevolg van stadige groei
- Konsentrasie van selle vir inokulasie: as die selle konfluent is teen die tyd wat hulle versamel word sal ‘n laer konsentrasie selle lei tot ’n hoër verdubellingsvlak.
- Temperatuur: laer temperatuur behoort te lei tot ‘n stadiger groei koers van die selle
- Medium voer-temperatuur: die voer-temperatuur van die medium sal geen beduidende verskil maak
- Belugting: die gebruik van “vent-caps” sal die suurstof inhoud van die roller bottels verhoog

Die volgende faktore is bestudeer vir hulle effek op die glukosilering tydens produksie:

- pH: beter glukosilering word verwag by by pH 6.9 dan by pH 6.7
- Perfusie koers: ‘n hoër perfusie koers sal lei tot beter glukosilering as gevolg van hoër glukose en glutamien konsentrasies

Die konsentrasie van die selle wat gebruik word vir inokulasie blyk die enigste faktor te wees wat die finale verdubbelingsvlak van die selle en die groei van die selle in die saad trein beïnvloed het. Die groei van die selle was beprek wanneer die selle konfluent geraak het en dus het ‘n laër sel konsentrasie by inokulasie gelei tot ‘n hoër sel verdubbelingsvlak. Dit word aanbeveel dat ‘n hoë sel konsentrasie by inokulasie gebruik word.

Die geleidelike toename van die finale verdubbelingsvlak van die selle in die saad trein is waarskynlik as gevolg van die variasie in sel tellings tussen verskillende operateurs eerder as as gevolg van die beheerde proses parameters. Dit word aanbeveel dat ‘n meganiese sel-teller gebruik word om die verskil in sel tellings tussen operateurs te kontroleer en om ‘n standaard te handhaaf tussen produksie lotte.

In die bioreaktors, het die pH geen beduidende invloed gehad op die glukosilering maar uit historiese data en die huidige data van hierdie eksperimente blyk albei die begin perfusie koers en die spesifieke perfusie koers ‘n belangrike invloed te hê op die glukosilering.

Laër vlakke van die begin perfusie koers lei tot beter glikosilsie en dit word aanbeveel dat elke produksielot ‘n begin perfusie koers het van 1.0 volume/dag. Die verhouding tussen die glukosilering en die spesifieke perfusie koers blyk om nie-liniêr te wees nie. Nog navorsing hieroor word aanbeveel, maar vir nou word dit aanbeveel dat die spesifieke perfusie koers onder 0.3 volumes/dag/10^9 selle gehou word. Hierde twee faktore blyk die oorsaak te wees vir die lae glukosilering wat in sekere produksielopies gevind was:

- RP33-133 : baie hoë spesifieke perfusie koers
- RP32-135 : hoë begin perfusie koers en baie hoe spesifieke perfusie koers
- RP32-138 : hoë begin perfusie koers
- RP33-139 : hoë begin perfusie koers

Dit word aanbeveel dat verdere navorsing gedoen word op die effek van die spesifieke perfusie koers asook die spesifieke koers van glukose verbruik en die spesifieke glutamien konsentrasie op die glukosilering van die produk.

ACKNOWLEDGEMENTS

Researching the production of recombinant proteins on an industrial scale proved to be quite an ambitious project, especially as one run (seed train and bioreactor) took a minimum of 60 days and could not be handled alone. It would not have been possible without the help of many people and I would like to acknowledge their contributions:

- Alain Desgeorges, Yannick Dumont and Emmanuelle Cameau, the OTS USP team at Merck Serono Aubonne for their generously shared process knowledge, for teaching me the ins and outs of large bioreactors, for their good spirits while processing over 90 roller bottles a day, for all the weekend work and for allowing me to run the project as I thought best.
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ABBREVIATIONS

ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
CHO	Chinese hamster ovary
D	Dilution volumes (volume/bioreactor volume/day)
DSP	Downstream processing
EPO	Erythropoietin
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
IFN-γ	Interferon-γ
LLC-PK_1	Procine kidney continuous renal cell line
mPL	Mouse placental lactogen
MRC-5	Normal Human Fetal Lung Fibroblast Cells
OK	Opposum kidney continuous renal cell line
PBS	Phosphate buffered saline solution
PD	Production day, number of days since production phase was started in a bioreactor
PDL	Population Doubling Level
r-tPA	Recombinant tissue-type plasminogen activator
USP	Upstream processing
v/v/d	volume medium/volume bioreactor/day
WD	Working day, number of days since the process was started
WI38	Human Fetal Lung Fibroblast cell

1 INTRODUCTION

Recombinant glycoproteins are important biopharmaceuticals, providing solutions for numerous previously untreatable illnesses, in everything from cancer to infertility. Most recombinant biopharmaceuticals are produced in mammalian cells due to their ability to provide the correct post-translational processing for use in humans. The post-translation processing influences many of the protein's properties including pharmacokinetics, bioactivity, secretion, half-life, solubility, recognition and antigenicity. The aim of this thesis is to study further the effects of certain controlled process parameters on the upstream production of a glycosylated recombinant protein produced by Chinese hamster ovary (CHO) cells at production scale within the confines of an existing process.

The process in question uses adherent CHO cells to produce a glycosylated recombinant hormone. As with most recombinant protein production processes, this process has two sections to the upstream production: a seed train section, to grow enough cells to inoculate the production bioreactor and a production section, which focuses on the production of a recombinant protein. In this process, the seed train is predominantly conducted in roller bottles, where the cells grow attached to the surface of the roller bottles, and production takes place in a perfusion bioreactor. In the perfusion bioreactors, the cells are attached to microcarriers and spin-filters are used for cell retention. The whole process uses medium with serum.

Two problems have been identified in the upstream section of the existing process:

1. The apparent gradual increase, over the past several campaigns, of the final population doubling level (PDL) of the cells at the end of the seed train (which must remain within certain specified limits for the cells to be used in production).
2. The low glycosylation levels of the product seen in certain campaigns (which must remain within certain specified limits for the final drug product to be used).

Determining the cause of these problems from historic data proved difficult. In previous campaigns, only a limited amount of data had been collected on the process. In the seed train, only the cell counts and viabilities were recorded. The rotational speed of the roller bottles, pH of fresh medium and temperature of the incubation chamber were checked periodically during and recorded as having remained within a specific permissible range. The metabolites, like glucose and lactate, were not recorded in the seed train data from previous campaigns.

The data recorded in production phase is more extensive than that in the seed train, however data on the glycosylation is limited. The only glycosylation data available is that of the final product bulks. During each run, fifteen harvests of the supernatant are collected. These harvests are then combined (sometimes from more than one run) and passed through several clarification, concentration, filtration and chromatographic steps in downstream processing (DSP) to form the final product bulks. The downstream processing is known to affect the glycosylation levels of the product. As the glycosylation is only measured after DSP, on the combination of several harvests, it is very difficult to shed any light on the effect the process conditions may have had on the glycosylation of the product using historic data.

Previous studies of the process have eliminated differences between runs in the raw materials used in the process and most other uncontrolled process parameters and have hypothesised that the solution to these problems lies within the ranges of the controlled process parameters of these sections of the process.

This study will focus on both sections of the upstream section of the process, each with different outcomes: the effect of the controlled process parameters on the cell growth and PDL will be investigated in the seed train and the effect of certain controlled process parameters on the glycosylation of the product will be investigated in the production phase.

2 LITERATURE SURVEY

Most large-scale industrial recombinant protein production processes based on mammalian, animal or insect cells have two parts to the upstream processing, a seed train or amplification phase followed by the production phase (Hughes and Hann, 2007), as is also the case in this process. The focus of the seed train is to grow enough cells to inoculate the production phase, while the focus of the production phase is, of course, the production of the desired protein. The literature study has been divided into two sections: the first section will focus on the possible effect of the controlled process parameters on the cell growth and final PDL during the seed train and the second section will focus on the possible effect of the controlled process parameters on the glycosylation of the product during production phase.

2.1 SEED TRAIN

In this process, the seed train is conducted in T-flasks and roller bottles. As the cells are adherent cells, they grow on the bottom of the T-flasks and on the sides of the roller bottles. The seed train is considered successful when it yields sufficient cells to inoculate a bioreactor, within a certain population doubling level (PDL) range, with a final viability greater than 80%.

The majority of the seed train is conducted in roller bottles; both 1750 cm^2 and 850 cm^2 nominal growth-area roller bottles are used, with the 1750 cm^2 roller bottles accounting for the majority of the process. The seed train takes place in five passages of five days each. The spent supernatant is removed and replaced with fresh medium two and four days after inoculation of each passage.

The following process parameters are controlled during the seed train:

- Medium volume
- Cultivation temperature
- Seeding density
- Rotational speed of the roller bottles
- Medium feed temperature

These parameters and their possible effects according to published scientific literature will be discussed in the following sections. The literature survey was limited to adherent, mammalian cells grown in either T-flasks or roller bottles.

2.1.1 MEDIUM VOLUME

The medium volume is used as a measure of the amount of growth medium added to each roller bottle.

Wu *et al.* (2005) used a factorial experimental design to study the effect of medium volume, seeding density, feeding frequency, medium type and serum concentration on the growth of MRC-5 cells in both roller bottles and T-flasks. After performing an ANOVA on their results with a 95% confidence level, they determined that increasing the medium volume had a positive effect on the PDL, i.e. using a greater medium volume lead to a higher PDL.

They proposed two possible theories for the increased PDL observed when increasing the medium volume. The first is that the increase in the total nutrient(s) availability at a greater volume (the medium composition was the same, so an increase in volume meant an increase in the total amount of nutrients) supported cell growth better. The second theory is that the lower oxygen tension at a higher medium volume, because of the increased diffusion length for gas exchange between the surface of the medium and the wall of the roller bottle where the cells were attached, allowed better proliferation of the cells. MRC-5 cells had previously been shown to proliferate better at a lower oxygen tension by Taylor *et al*. (1978) and Balin *et al*. (1984) as cited by Wu *et al*. (2005). Though no definitive theory has been proposed for this effect, it would appear that “oxygen regulates the growth of human cells under pressures of oxygen physiologic to humans” and that oxygen toxicity at higher oxygen tension leads to lower cell growth, though this effect is also dependent on the cell density and is more apparent at low cell densities.

Ryan *et al.* (1975), who worked exclusively on the cell culture volume with WI38 cells in T-flasks, found similar results, showing that doubling the cell culture volume gave a two-fold increase in the cell yield. They attributed this to the increased total amount of serum growth factor(s) present at greater medium volumes.

Several authors have investigated the effect of nutrient concentration on cell growth (Hayter *et al*., 1991 and Lu *et al.*, 2005), but Gstraunthaler *et al*. (1999) investigated the influence of both cell culture medium volumes and glucose concentration on renal epithelial cells (LLC-PK_1 cell line) in roller bottles. Gstraunthaler *et al.* (1999) did not discuss the cell growth rates but they found that changes in both the nutrient supply and culture medium volumes significantly influenced the metabolic rates and the levels of enzyme activity, both of which increased with increasing nutrient supply and culture volume. Both glucose supply and medium volume were determining factors in the glycolytic rates of LLC-PK_1 cells.

Given the work of these research groups, it appears that the medium volume has an effect on the cell growth. This could be either because of the change in the availability of nutrients or for another reason, like the change in oxygen tension as suggested by Wu *et al* (2005). No literature could be found on the effect of a slight decrease in oxygen tension on CHO cell proliferation. The available literature appears to focus on significantly decreased oxygen content where the cell culture conditions are oxygen-limiting (Lynn *et al.*, 1992).

In this process, the medium composition is fixed, so the effect of glucose on the CHO cells in this process at fixed medium volumes cannot be investigated. However, the effect of medium volume can be investigated and vent caps, which allow for passive aeration, could be used to help distinguish the effect of the medium volume from the effect of the oxygen concentration.

2.1.2 Cultivation Temperature

The cultivation temperature is the temperature of the incubation chamber in which the cells are grown.

Cultivation temperature is an important cell culture parameter (Sonna *et al.*, 2002; Tsao *et al.*, 1992) and is indeed used in the production phase of many cell culture processes to prolong the experimental and stationary growth phases (Rössler B. *et al*., 1996). Several authors have studied the effect of temperature on cell growth in roller bottles but one of the most extensive studies was performed by Tsao *et al.* (1992). Tsao *et al*. (1992) studied the effect of wide range of incubation temperatures (32 – 42 °C) on recombinant human EPO producing CHO cells grown in roller bottles. Within the range of 36 °C – 38 °C, they found that the cells had a higher growth rate at 38°C (determined microscopically) which lead to rapid medium acidification and severe cell detachment towards the end of the process. In the range of 32 – 37 ° C, there were no noticeable differences in cell morphology. Incubation at 42 °C resulted in a steep decline in viability without further cell growth.

The temperature range of the present process under consideration is specified as 35 – 37 °C. Given that Tsao *et al*. (1992) found a difference in growth rates over a 2 °C temperature difference, just 1 °C higher than that of the process, it is possible that there will be a difference in growth rate between 35-37°C, which could lead to a difference in PDL.

2.1.3 Seeding Density

The seeding density is the number of cells used to inoculate a roller bottle at the beginning of a passage (measured in viable cells/roller bottle).

Both Wu *et al*. (2005) and Tsao *et al*. (1992) showed that the seeding density has an important effect on cell growth. High seeding densities seem to result in faster depletion of nutrients, higher cell detachment and more debris and cells in suspension. Lower seeding densities do not appear to lead to these problems within the same period. In that sense, lower seeding densities can be seen as advantageous.

However, the results of Wu *et al*. (2005) show that lowering seeding density can have a positive effect on the PDL, i.e. lowering the seeding density lead to a higher PDL. They also showed that contact inhibition at higher cell densities appeared to limit cell growth in their cell line once cells reached confluence.

By contrast, an internal study, also performed with CHO cells (105923, 2006), showed that using a low seeding density allowed researchers to harvest cells while still in exponential phase, i.e. cell growth was not inhibited at higher densities. It is uncertain which effect the seeding density would have on this process but varying the seeding density would allow one to determine whether cells have reached confluence or are still in exponential growth phase when they are harvested and what the effect of this would be on the final PDL.

2.1.4 Rotation rate

The rotational rate is the speed at which the roller bottles rotate on the rollacell.

In their extensive study, Tsao *et al.* (1992) also studied the effect of rotation rate on CHO cells producing recombinant human EPO in roller bottles. They found that in varying the rotational speed in the range of 0.2 - 1.0 rpm, while keeping the temperature constant at 37 °C, had no noticeable effect. It was only once the rotational speed was further increased to 2.0 rpm that differences in the cell culture were noted. This agrees with other authors (Rodriguez *et al.*, 2005), and it seems that approximately 1 rpm difference is required to make a significant difference. The seed train specifications only allow for a difference of 0.1 rpm in rotation rate (0.3 - 0.4 rpm), ten times smaller than that required to make a significant difference to the cell culture. The rotation rate of the roller bottles is regularly checked throughout the process and unlikely to change enough to make a significant difference to the cell culture.

2.1.5 ADDITIONAL PROCESS CONSIDERATIONS

This section will discuss additional considerations do to with the operation of the process and controlled process variables not discussed in literature.

Medium volume

Medium volume is the volume of medium in the roller bottle

In the process under investigation, 300 ml of growth medium is used in the smaller roller bottles and 600 ml of growth medium is used in the larger roller bottles. This differs from the volume recommended by Corning, the roller bottle manufacturer. Corning recommends 170 – 255 ml of medium for the smaller 850 cm^2 roller bottles and 350 - 525 ml for the larger 1750 cm^2 roller bottles (Corning Roller Bottles - Selection and Use Guide, 2005).

Cultivation temperature

The cultivation temperature is the temperature of the incubation chamber in which the cells are grown.

The temperature range of the present process under consideration in this thesis is specified as 35 – 37 °C. Given that Tsao *et al*. (1992) found a difference in growth rates over a 2 °C temperature difference, just 1°C higher than that of the process, it is possible that there will be a difference in growth rate between 35 – 37 °C, which could lead to a difference in PDL.

The temperature in the incubation chamber is well controlled and a study of the temperature mapping of the incubation chamber during a seed train shows a maximum difference of 0.9 °C between different areas in the chamber due to operators opening the door to the incubation chamber to retrieve the roller bottles for the necessary medium changes. The effect of both the permitted temperature range 35 – 37 °C and the maximum measured difference 35 - 36 °C could be investigated.

Medium feed temperature

The medium feed temperature is the temperature of the fresh medium when it is added to the roller bottles.

In this process, medium at 36 °C is used to inoculate roller bottles and for medium changes. The feed temperature of medium is not a point that is often addressed in literature. A literature search yielded no results on this specific topic, though the standard procedure is to add pre-heated medium to the cell culture. From pre-tests, it was found that the medium took approximately four hours to warm from storage temperature (2 - 8 °C) to 36 °C. To determine whether these four hours make a difference to the cell culture, the medium temperature will also be tested.

2.1.6 SUMMARY OF POSSIBLE EFFECTS FROM LITERATURE

From this literature survey, there appear to be three controlled process parameters that could affect the cell growth within their specified ranges: the medium volume, cultivation temperature, seeding density. Depending on the level of confluence reached by the cells, decreasing medium volume might yield a decrease in the PDL; decreasing the seeding density might yield an increase in the PDL, while decreasing the temperature might decrease the growth rate, and thus the PDL.

Two other parameters will also be investigated: the feed temperature of the medium and the use of vent caps. The use of vent caps, which allow for passive aeration, will help distinguish between the effect of the medium volume and the oxygen concentration and investigating the medium feed temperature will help determine whether it is necessary to pre-heat the medium.

2.2 BIOREACTOR PROCESS

Usually, a study of a recombinant protein production method focuses on increasing the titre of the product. However, in this case the titre is considered satisfactory and the problem lies with the level of glycosylation; specifically the extent of the sialylation (the addition of sialic acid to the oligosaccharides attached to the protein) of the product. The literature review of the production phase of the process will first discuss glycosylation as background to the problem and then discuss the possible influence of controlled process parameters.

In this process, production takes place in perfusion bioreactors using adherent CHO cells that grow on microcarriers with spin-filters for cell-and-microcarrier retention. The temperature, dissolved oxygen, speed of the impeller, speed of the spin-filter, pressure and volume of liquid in the reactor are all automatically controlled. The pH is also automatically controlled with the addition of CO_2 or sodium hydroxide, as required. The concentration of the metabolites (glucose, lactate, glutamine, etc.) is dependent on the perfusion rate, which is controlled manually. The perfusion rate is specified at the start of the production phase and is then increased as is needed to keep the glucose concentration and the lactate concentration within certain specifications. The seeding density is also specified.

2.2.1 GLYCOSYLATION AND RECOMBINANT PROTEINS

Glycosylation is an enzymatic process where glycans (linked saccharide molecules) are attached to proteins.

Recombinant protein therapeutics have transformed modern medicine over the last decade, providing therapies for a multiplicity of previously untreatable illnesses ranging from cancer to infertility. Recombinant proteins are generally produced through large-scale cultivation of genetically engineered cells that contain the transfected genes for the proteins of interest (Chu and Robinson, 2001 and Jayapal *et al.,* 2007). Most recombinant proteins for pharmaceutical applications are produced in mammalian cells because of their capacity for proper protein folding, assembly and post-translational modifications (Restelli and Butler, 2002 and Wurm, 2004). Several mammalian cell lines are used to produce recombinant proteins, but by far the most prominent are Chinese hamster ovary (CHO) cells, which are used to produce nearly 70% of all recombinant proteins (Wurm, 2004 and Jayapal *et al.*, 2007).

The post-translational modifications of a recombinant protein are vital to the overall therapeutic profile of a recombinant protein. One of the most important post-translational steps of a recombinant protein is the glycosylation. Many of the pharmacological properties of a recombinant protein including the pharmacokinetics, bioactivity, secretion, half-life, solubility, recognition and antigenicity are determined by the glycosylation of the protein (Rasmussen, 1992, Restelli and Butler, 2002 and Werner *et al*. 2007).

Glycosylation is the addition of carbohydrates (called glycans or oligosaccharides) to synthesised proteins (Butler, 2005); the glycosylated proteins are produced as pools of different glycoforms (varying glycan structures attached to the same protein backbone). The variation in the structure of the attached glycans is dependent on the presence of various glycosyltranferase enzymes in the Golgi, and the competition of these enzymes for the same substrate (Khmelnitsky, 2004).

There are three types of glycosylation: N-glycosylation, O-glycosylation and GPI-glycosylation. In N-glycosylation, the glycan is bound to the protein, via an N-glycosidic bond to an Asn residue, within the consensus sequence Asn-Xxx-Ser/Thr. In O-glycosylation, the glycans are added to an exposed Thr/Ser residue on the completely folded protein post-translationally via an O-glycosidic bond. In GPI-glycosylation, the glycophosphatidylinositol (GPI) anchor is used to attach certain proteins to cell membranes (Jenkins *et al*., 1996; Restelli and Butler, 2002). As the protein in question has only N-glycosylation, this study will focus only on N-glycosylation.

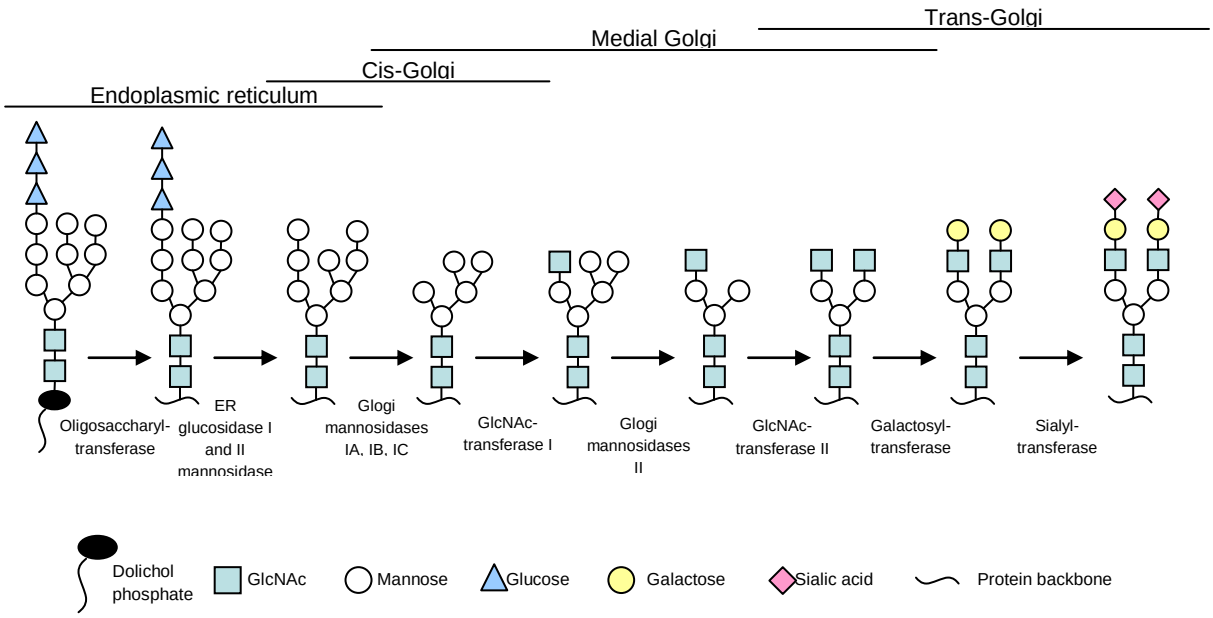

FIGURE 1. ILLUSTRATION OF THE BIOSYNTHESIS OF N-LINKED GLYCANS, ADAPTED FROM RESTELLI AND BUTLER (2002).

N-linked glycosylation starts in the endoplasmic reticulum (ER), as illustrated in Figure 1, where the precursor oligosaccharide is added to the protein by the oligosaccharyltransferase enzyme (all N-linked glycans share the same core structure: $Man_3GlcNAc_2$-Asn). This is followed by a series of trimming steps performed by the glucosidase enzymes before the newly synthesised glycoprotein is transported to the Golgi by vesicles. In the Golgi a series of trimming and addition steps take place, performed by glycosidase and glycosyltransferase enzymes, until the final processing steps which involve the addition of sialic acid, by α2,3-sialyltransferase and α2,6-sialyltransferase, poly-N-acetyl lactosamine, by β N-Acetylglycosaminyltransferase, or fucose by α1,6 fucosyltransferase (Restelli and Butler, 2002). The final product is then secreted by the cells into the supernatant.

As it is not possible to monitor the glycosylation in detail, the extent of glycosylation in this process is monitored by determining the extent of sialylation. As illustrated in Figure 1, sialylation, the addition of sialic acid to the glycan by the sialyltransferase enzymes, is one of the final steps of the glycosylation process. The extent of sialylation is determined by the charge profile (the percentage of neutral, mono-, di- and tri-sialylated glycans), which is characterised by the Z-number.

The glycosylation of a recombinant protein can be affected by many aspects of the production process including the host cell line, the method of culture, the extra-cellular environment and the protein itself (Jenkins *et al.*, 1996 and Restelli and Butler, 2002). Working with an existing process means that only the extra-cellular environment can be changed, as the host cell line, the method of culture and the protein itself have already been specified. The next sections will focus on the controlled process parameters, their permissible ranges and the possible effect they could have on the extent of glycosylation.

As glycosylation is specific to cell line, cell type and cultivation method, the literature study will be limited to adherent CHO cells cultivated in a serum-containing environment in bioreactors.

2.2.2 TEMPERATURE

The temperature is the temperature inside the bioreactor during production.

Cultivation temperature is an important cell culture parameter and is often used in the production phase of many cell culture processes to prolong the production phase (Rössler *et al.*, 1996). There are many varying opinions about the effect of temperature on the glycosylation of a recombinant protein. Yoon *et al.* (2003) found that the glycosylation of EPO produced from CHO cells at 33 °C was comparable to that produced at 37 °C. Bollati-Fogolin *et al.* (2005) reached the same findings when they tested the same temperature shift on CHO cells producing recombinant human granulocyte macrophage colony stimulating factor. However, Trummer at al. (2006b) found reduced sialylation of EPO-Fc when they compared production at 37 °C to production at 33 °C and 30 °C, also using CHO cells. While Woo *et al.* (2008) tested a range of temperatures between 25-37 °C using EPO-producing CHO cells, and found that the glycosylation of the product was comparable between 32 - 37 °C, but was negatively affected below 32 °C.

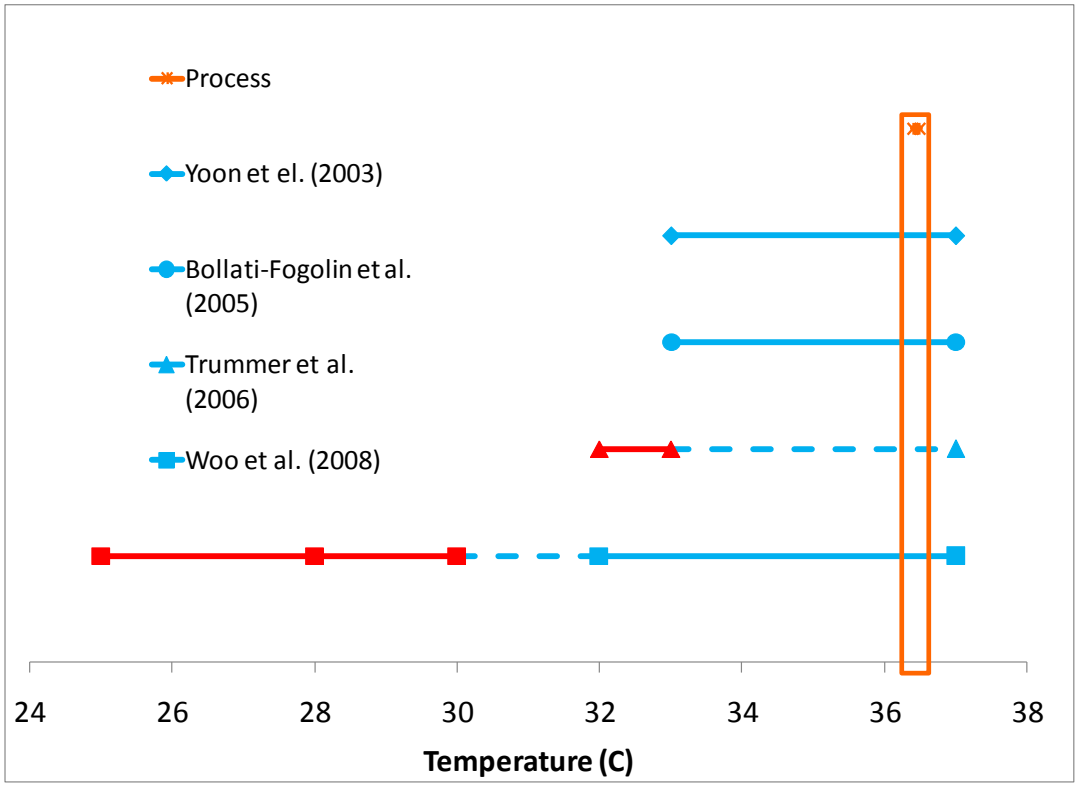

FIGURE 2. EFFECT OF TEMPERATURE ON GLYCOSYLATION FROM LITERATURE (BLUE INDICATES SATISFACTORY GLYCOSYLATION, RED INDICATES UNSATISFACTORY GLYCOSYLATION AND ORANGE INDICATES THE PROCESS SPECIFICATIONS).

Yoon *et al.* (2003) and Bollati-Fogolin *et al.* (2005) simply stated that the cultivation temperature did not appear to have an effect on the glycosylation of the proteins they were investigating, while both Trummer *et al.* (2006b) and Woo *et al*. (2008) found that there was a corresponding increase in specific production rate at decreased temperatures. They suggested that a higher specific production rate meant that the protein would spent less time in the Golgi and that there would therefore be less time for glycosylation which is what lead to the decrease in glycosylation levels. Another possibility, which does not appear to have been considered, is that the enzymatic activity of the enzymes involved in glycosylation could decrease at lower temperatures.

However, despite the conflicting opinions and differences between cell lines, it would seem that between 35 °C and 37 °C, there appears to be no great effect on the cells (Trummer *et al*. 2006a) or on the product quality. The permissible temperature range during production phase in this process is 36.4 °C - 36.6 °C. It appears highly unlikely that a temperature difference of 0.2 °C will lead to differences in glycosylation of the product, as can be seen from

2.2.3 pH

The pH is the pH of the supernatant in the bioreactor during production.

pH is another important cell culture parameter as the enzymes involved in glycosylation are often pH sensitive. The external pH can have an effect on the internal pH of the cell, which can result in the reduction of key glycosylation enzymes (Rothman *et al.*, 1989).

Borys *et al.* (1993) performed an extensive study of the effect of pH on mPL-producing CHO cells. They found that the glycosylation was pH dependent with maximum glycosylation between pH 6.9 - 8.2 and decreasing levels outside this range. Yoon *et al.* (2003) found maximum glycosylation in the range of pH 6.8 - 7.2 for EPO producing CHO cells, while Trummer *et al.* (2006b) preferred pH 6.9 - 7.1. The permissible pH range for the process under investigation is 6.7 - 6.9, which straddles the lower end of the ranges found to give maximum glycosylation, as seen in Figure 3. Experiments will be carried out to determine whether the changes in pH of the process affect the sialylation levels of the protein.

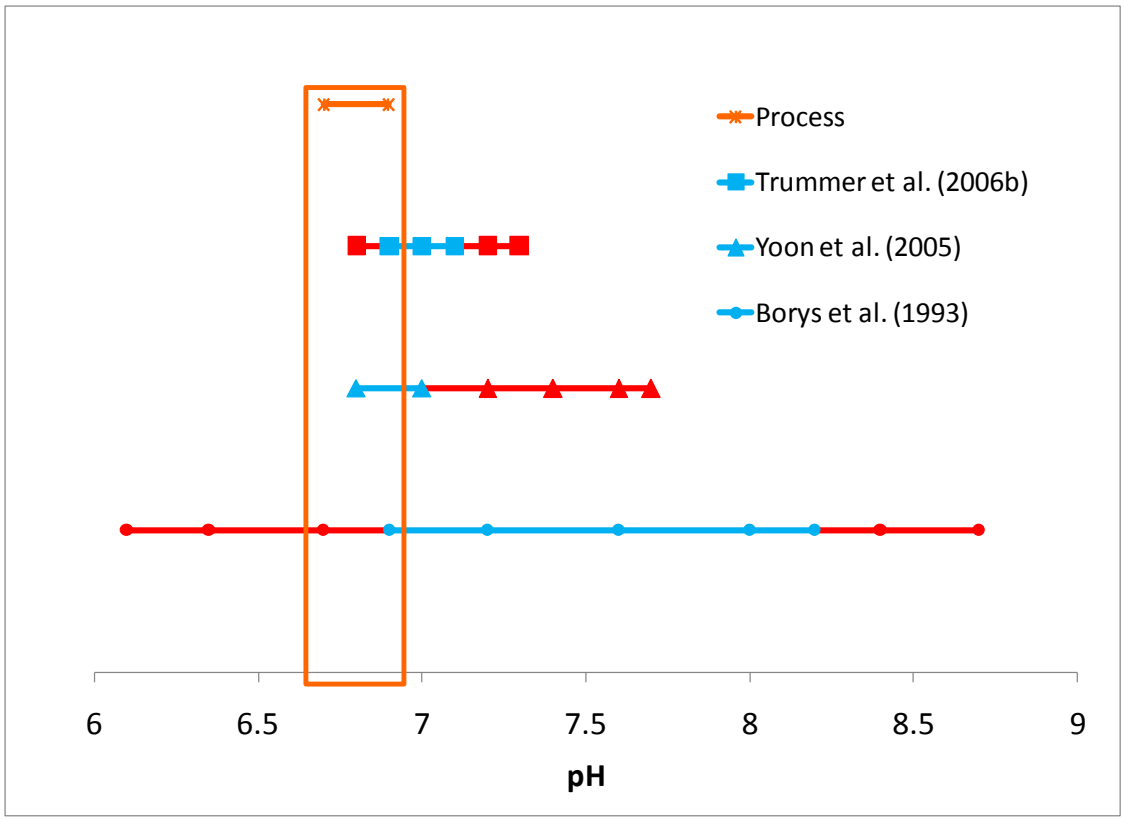

FIGURE 3. EFFECT OF PH ON GLYCOSYLATION FROM LITERATURE (BLUE INDICATES GOOD SATISFACTORY GLYCOSYLATION, RED INDICATES UNSATISFACTORY GLYCOSYLATION AND ORANGE INDICATES THE PROCESS SPECIFICATIONS).

2.2.4 METABOLITES

The metabolites are any substances in the supernatant which are either necessary to sustain the cells or are produced by the metabolic processes of the cells.

Glucose and Glutamine

Glucose and glutamine are the cells' main energy sources as well as the precursors to a number of intermediates necessary for protein synthesis and glycosylation. Their ambient concentrations often affect the degree of glycosylation of a product as Hayter *et al.* (1992 and 1993) showed with IFN- γ producing CHO cells. More recently, Wong *et al.* (2005) showed that glutamine concentrations below 0.1 mM or glucose concentrations below 0.7 mM could lead to decreased sialylation for IFN-γ producing CHO cells. All studies theorised that the decrease in glycosylation was due to a lack of the substrates required by the glycosylation enzymes to complete glycosylation.

In this process, the lowest permitted glucose concentration is 8.3 mM, so according to the available literature there should be sufficient glucose to function as precursors for glycosylation, as seen in Figure 4. However, the glutamine concentration is not usually monitored and it may prove to have an effect.

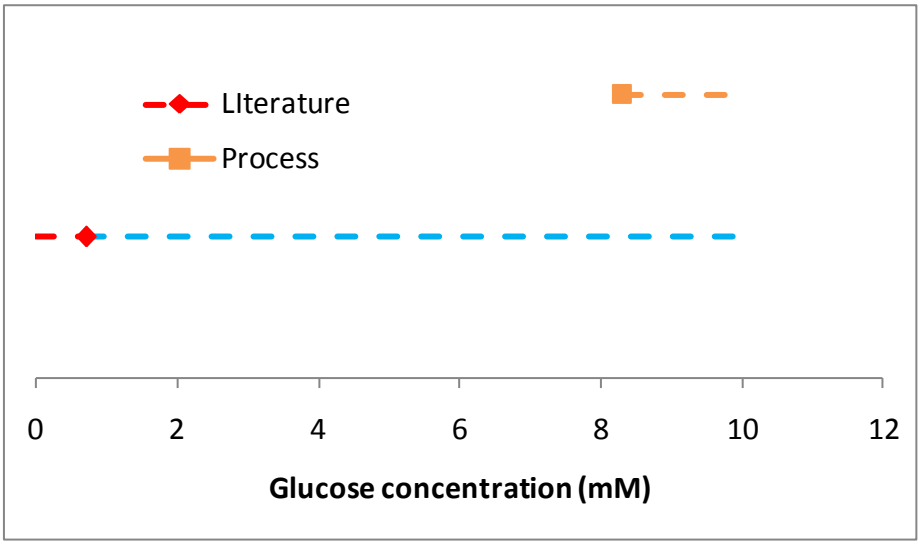

FIGURE 4. GLUCOSE CONCENTRATION, RED INDICATES UNSATISFACTORY SIALYLATION, BLUE INDICATES SATISFACTORY SIALYLATION AND ORANGE INDICATES THE PROCESS RANGE.

Lactate and ammonium

Lactate and ammonium are waste products, produced by the cell during glucose and glutamine consumption, respectively. Nothing in literature suggests that the lactate concentration has an effect on the glycosylation. However, the ammonium concentration appears to have a negative effect on the glycosylation through the inhibition of galactosyltransferase and sialyltransferase enzymes that affect the galactosylation and sialylation steps of the glycosylation process as shown by Chen *et al. (*2006).

Andersen *et al.* (1995) found that it was preferable to keep the ammonium concentration in cultures below 2 mM, while Borys *et al*. (1993) found that the glycosylation of CHO-produced mPL-I was inhibited in the range of 3 mM – 9 mM. Borys *et al*. (1993) also proved that the inhibition of glycosylation in the presence of ammonium could not be contributed to the osmolarity or the extra-cellular events that occur after the secretion of the glycoprotein. The maximum ammonium concentration that typically occurs in this process is approximately 3.5 mM. It would be interesting to see whether this has an effect on the glycosylation of the product.

Perfusion rate

The perfusion rate is the rate at which supernatant is harvested from the bioreactors and fresh medium is added to the bioreactors.

The concentration of metabolites in the process can be controlled through either the medium composition or the addition of fresh medium. In this process, the medium composition is fixed, so the concentration of metabolites has to be controlled through the addition of fresh medium, which is measured as the perfusion rate. Normally, the initial perfusion rate is set, and then the perfusion rate is increased as is required to keep the glucose concentration above 1.0 g/L and the lactate concentration below 1.5 g/L.

At its extremes, the perfusion varies from approximately 1 L/hour to 2 L/hour, which could influence the hydrodynamic forces on the cells in the bioreactor. Cells on microcarriers are more sensitive to hydrodynamic forces than freely suspended cells (Papoutsakis, 1991). Hydrodynamic stress on cells grown on microcarriers happens as either: collisions between microcarriers, collisions with parts of the bioreactors or interactions with turbulent eddies the size of the microcarriers. These eddies are influenced by agitation and perfusion rate. Very high stress would destroy the cell wall, while lesser degrees of stress can have varying effects including reduced growth, apoptosis and increased protein production (Czermak *et al.* 2009).

Senger and Karim (2003) investigated the effect of shear stress on the production and glycosylation of r-tPA produced by CHO cells in suspension cell culture. They found that at moderate levels of shear stress the total production of r-tPA was maximised. While at damaging levels of shear stress, the level of glycosylation was at its lowest, probably due to the decreased residence time of the protein in the endoplasmic reticulum (ER) because of increased protein synthesis caused by the shear stress protection mechanisms of the cell. As cells on microcarriers are more sensitive to shear stress than cells in suspension, it may be that the stress on the cells due to increase in perfusion rate will have a similar effect.

2.2.5 Oxygen and Carbon Dioxide

The oxygen and carbon dioxide are the concentrations of oxygen and carbon dioxide in the bioreactor during production.

The dissolved oxygen content of the medium can also affect the glycosylation levels, though most authors agree that at dissolved oxygen levels of 10 - 90% the glycosylation is stable. It is only when the dissolved oxygen concentration goes outside these limits that the glycosylation begins to vary (Chotigeat *et al.*, 1994, Butler, 2005 and Restelli *et al.*, 2006).

The dissolved oxygen content of the medium is controlled at 40 ± 10%, well within the apparent "safe" range, as seen in Figure 5. It is probably not the cause of the low glycosylation of the product see in certain batches of final drug product.

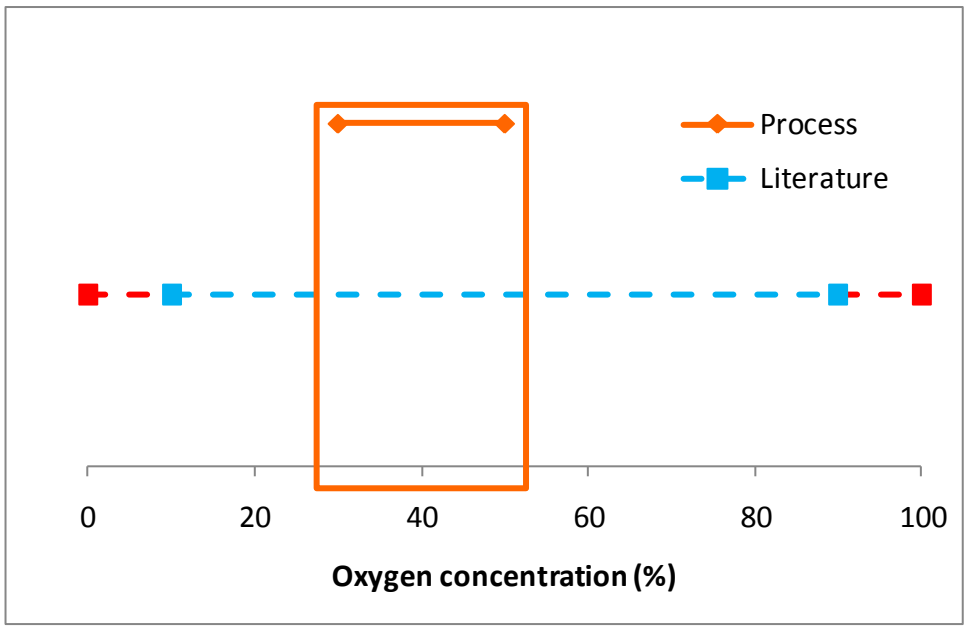

FIGURE 5. EFFECT OF OXYGEN CONCENTRATION ON GLYCOSYLATION (BLUE INDICATES SATISFACTORY GLYCOSYLATION, RED INDICATES UNSATISFACTORY GLYCOSYLATION, ORANGE INDICATES PROCESS SPECIFICATIONS).

The carbon dioxide content of the culture does not appear to influence the glycosylation levels, except at very high levels. Kimura and Miller (1997) found a 40% decrease in the extent of sialylation from 36 mmHg CO_2 to 250 mmHg CO_2. In this process, the CO_2 tends to remain below 100 mmHg CO_2. It is also not directly controlled so cannot be manipulated during the culture, though it can be monitored

2.2.6 SPEED OF THE IMPELLER, SPEED OF THE SPIN-FILTER, PRESSURE AND VOLUME OF BIOREACTOR

No literature could be found on the effect of the spin-filter speed, pressure or volume of the bioreactor on the glycosylation of the product, though increasing the impeller speed would increases the shear stress on the cells, which has been shown to have a negative effect on the glycosylation. However, all these parameters are strictly controlled and there is no significant variation during runs or between different runs. It seems unlikely that they are the cause of the low glycosylation levels seen in certain batches of final drug product.

2.2.7 TIME IN CULTURE

The time in culture is the amount of time the cells spend in production phase.

The glycosylation of recombinant proteins has been shown to change over time in cell culture. In batch processes this is often related to the decrease in glucose and glutamine concentration (Hayter *et al.,* 1993 and Nyberg *et al*. 1999) but it has also been shown to be glucose- and glutamine-independent during continuous processes as illustrated by Curling *et al.* (1990). Curling *et al*. (1990) found that the decrease in glycosylation over time was related to the decreased specific growth and production rates of CHO-produced IFN-γ. A third possibility is the degradation of the glycan chains in the cell culture after secretion by extracellular glycosidase (Gramer, 1999). An internal study (LU-P-00009, 2005) determined that the glycosidase activity in the supernatant of the process in question was too low to be significant, eliminating degradation of the glycoproteins because of glycosidase activity.

Unfortunately, there is very little historic data on the change in glycosylation over time in culture for this process, as most historic data is for final bulks (combinations of several harvests) and therefore no *a priori* assumptions can be made about it. The production time in cell culture is fixed, however if a trend exists to indicate the change of the glycosylation over the course of the culture it might be possible to make recommendations regarding the mixing of harvests, to ensure the highest Z-number in the final bulks.

2.2.8 SEEDING DENSITY

The seeding density is the number of cells used to inoculate the bioreactors.

The concentration of the cells in the inoculum is allowed to vary from 12 – 18 x 10^9 viable cells per bioreactor. However, the range of seeding density has already been tested internally (LU-P-00010, 2006) and found to have an insignificant effect on the glycosylation. No literature could be found that addressed the effect of the seeding density on the glycosylation.

2.2.9 SUMMARY OF POSSIBLE EFFECTS

Glycosylation of recombinant proteins is highly tissue-type and cell-line specific. Even in the same cell line, there are usually differences due to the protein produced (as glycosylation sites vary from one protein to another) and the expression-system used (Werner *et al.* 1998; Gawlitzeck *et al*., 1995). This means that for most processes the influence of production parameters has to be specifically determined for that process. However, there appears to be certain general trends required for good glycosylation:

- The availability of sufficient substrates from which to form glycans
- Sufficient time in the ER and Golgi to allow for more complex glycosylation

- Favourable conditions in the ER and Golgi (especially pH) to allow the all enzymes involved in glycosylation to function correctly

From this review, there appear to be two controlled process variables that are likely to affect the glycosylation of the product within the confines of the process specifications. The process variables are the pH and the perfusion rate.

The permissible pH range straddles the lower end of the pH range recommended for maximum glycosylation from literature and it is possible that the variations in pH during the production phase are responsible for the unsatisfactory glycosylation found in certain final bulks. The perfusion rate has not been shown from literature to have a direct influence on the glycosylation. However, it is the only way to control the glucose and glutamine concentrations and it may also have an influence on the hydrodynamic forces the cells are subject to which have been shown to influence the glycosylation.

3 PROBLEM STATEMENT AND THESIS OBJECTIVES

There are two process challenges regarding an existing recombinant-protein production process:

1. The gradual increase, over the past several campaigns, of the final population doubling level of the cells (which must remain within certain specified limits) at the end of the seed train.
2. The low glycosylation levels of the product seen in certain campaigns, which meant that a certain number of final product batches where below the specified acceptable glycosylation limits.

Previous studies of the process have eliminated differences between runs in the raw materials used in the process and most other uncontrolled process parameters. It has been hypothesised that the solution to these problems lies within the ranges of the controlled process parameters of these sections of the process. Following a survey of the available literature on these sections of the process, several process parameters were chosen for investigation.

In the seed train, the aim of this study was to determine the influence of the controlled process parameters on cell growth and the final PDL. The medium volume, seeding density and cultivation temperature were chosen for investigation.

From literature, it is hypothesised that their effects would be as follows:

- Decreasing the medium volume will yield a lower PDL due to slower cell growth caused by lower glucose availability.
- Decreasing the seeding density will yield a higher PDL.
- Decreasing the temperature will decrease the growth rate and yield a lower PDL.

There are two other parameters that will also be investigated in the seed train: the feed temperature of the medium and the use of vent caps.

It is hypothesised that their effects will be as follows:

- There will be no significant difference to the cell growth when cold medium is used rather than pre-heated medium.
- Using vent caps will increase the oxygen content of the medium in the roller bottles and the cell growth, yielding a higher PDL.

In production phase, the aim is to determine the influence of the controlled process parameters on the glycosylation of the protein during production. The pH and perfusion rate were chosen for investigation and the following hypotheses were made for their effects:

- better glycosylation will be seen at pH 6.9, than at pH 6.7
- a higher perfusion rate will lead to better glycosylation due to increased glucose and glutamine availability

4 Materials and Methods

4.1 The Cells

CHO VI C6 cells transfected with the gene to express a certain recombinant hormone were used for this study. The name of the hormone and the source of the DNA are confidential.

These cells are adherent cells. Therefore, they are grown on the surface of the roller bottle in the seed train and on microcarriers in the bioreactors during production.

4.2 Amplification process

4.2.1 The process

The amplification process consists of five, five-day passages. On working day 0, roller bottles (*Corning*) are inoculated with cells and medium. Basic medium containing fetal bovine serum (FBS) was used. The exact medium composition is confidential. On working day 2 and working day 4, the spent medium was removed and replaced with fresh medium, called a medium change. On working day 5, the final day of the passage, the cells are detached from the roller bottle by trypsinisation.

For trypsinisation, the spent medium was removed and the roller bottles were washed with PBS. The cells were then exposed to trypsin for 5 minutes to detach them from the sides of the roller bottle. After 5 minutes, the trypsin was neutralised with fresh medium and the detached cells were pooled and used to inoculate the next passage.

All passages took place in incubation chambers at 36°C. The roller bottles were placed on rollacells, large racks of rotating rollers that roll the roller bottles. The rotational speed of the roller bottles was 0.3-0.4 rpm. The speed of the rollacells was checked after each medium change to ensure that the rotational speed of the roller bottles remained within the specified range.

4.2.2 EXPERIMENTAL DESIGN

Experiments on the amplification process were conducted in two sections. Initially, a screening experiment was performed using 850 cm^2 roller bottles to determine which factors and interactions had the most significant influence on the cell growth. These factors were then tested in the 1750 cm^2 roller bottles, which make up the majority of the process.

For the screening experiment, a full factorial design with five factors on two levels was used; the choices for the high and low levels are discussed in the following paragraphs. The low and high levels were chosen at the outlying ends of the range considered practical to increase the probability that any effect they had on the process would standout beyond the normal process variation.

Medium volume

Corning, the manufacturer of the roller bottles used in this process, recommends a range of 170 – 255 ml of medium for 850 cm^2 roller bottles and 350 - 525 ml for 1750 cm^2 roller bottles (Corning Roller Bottles - Selection and Use Guide, 2005). However, the process specifications stipulate that 300 ml and 600 ml medium should be used in the 850 cm^2 and 1750 cm^2 roller bottles, respectively.

Operators add medium to the roller bottles by pouring it into the bottles and measure the volume according to the scale on the roller bottle. This often occurs at an oblique angle, especially in the taller 1750 cm^2 roller bottles, and there is frequently some variation in the amount of medium in roller bottles. To test whether this leads to differences in the PDL, the effect of the minimum amounts suggested by Corning and the full amount specified in the process specifications were compared.

Aeration

Aeration was tested as either an open or a closed system. An open system is defined as roller bottles with non-wetting vent caps that allow gas exchange. Whereas a closed system is defined as roller bottles with the standard caps supplied with the roller bottles that do not allow gas exchange.

In order to better follow the effect of the vent caps, Planar Oxygen Minisensors (*Presens, Germany*) were attached to the inside of the 850 cm^2 roller bottles which allow the measurement the pO_2 in both the medium and the air above the medium in the roller bottles without having to open the roller bottle and thus disturb the system. The sensors were not used on the 1750 cm^2 roller bottles due to time constraints. External pO_2 measurements of the pO_2 in the spent medium were also taken on the ABL5 (*Radiometer, Denmark*) at each medium change as a comparison.

Temperature

To test the effect of temperature and verify whether it has an effect on the cell culture, the 2°C temperature difference (35-37°C) was tested as part of the screening study in 850 cm^2 roller bottles. When the effect proved to be significant, the 1°C actual temperature difference (35-36°C), observed in the incubation chambers during temperature mapping, was tested in 1750 cm^2 roller bottles.

Seeding Density

As the 850 cm^2 roller bottles that were used in this study have half the nominal growth area and require half the volume of medium of the 1750 cm^2 roller bottles. As the seeding density for the 850 cm^2 roller bottles is not specified in the amplification procedure – it simply states that all the cells obtained from the T-flasks should be used - half the number of cells will be used for the seeding density. The lower seeding density was $1/5^{th}$ of the original seeding density, as had previously been tested in an internal study. The levels tested for the 850 cm^2 roller bottles were therefore 75-100 x 10^6 cells per roller bottle and 20-25 x 10^6 cells per roller bottle.

Medium Feed temperature

To test the impact of the medium feed temperature on cell growth and attachment, the medium used in these experiments was either warmed overnight to the specified temperature range of 35 - 37°C or taken directly from storage and used at approximately 6°C. The medium storage temperature is 2 -8 °C.

Interaction between process variables

Interaction effects occur when several factors (process variables) are important in combination. The more factors involved, the higher the order of the effect (2 factors is a second order interaction, 3 factors a third order interaction, and so forth). As cell amplification in roller bottles can be considered a relatively simple process, with relatively few parameters that can be directly controlled, it can be assumed that higher order interactions will be negligible. Indeed, the highest order interaction effect that has been reported for a roller bottle process in literature is a second order interaction (Wu *et al.*, 2005). Based on this assumption all first order interactions were repeated in triplicate, second order interactions were repeated in duplicate, and third order interactions were performed only once as a to check for possible interactions. Fourth and fifth order interactions were ignored. Analysis of variance (ANOVA) was performed on results, with an alpha of 0.05. See the appendix for complete ANOVA results.

A summary of the values used for the levels can be seen in Table 1. The normal column gives the values of the base case against which these changes will be compared. The changes for each factor are highlighted in Table 1.

TABLE 1. VALUES USED FOR HIGH AND LOW LEVELS OF FULL FACTORIAL EXPERIMENT IN 850 CM^2 ROLLER BOTTLES. NORMAL REPRESENTS THE BASE CASE AGAINST WHICH THE CHANGES HIGHLIGHTED IN BLUE WERE COMPARED.

Factor	(+) level	(-) level	Normal
Aeration	Vent cap	Closed cap	Closed cap
Medium Volume	300 ml	170 ml	300 ml
Seeding density	75-100 x 10^6 cells	20-25 x 10^6 cells	75-100 x 10^6 cells
Feed Temperature	2-8°C	37°C	37°C
Cultivation Temperature	35°C	37°C	37°C

The next set of experiments, performed in the larger 1750 cm^2 roller bottles, focused on only three factors and did not include any interactions, as they had been proven insignificant. The factors and levels used can be seen in Table 2. The (-) level also corresponds to the base case against which these changes were compared.

TABLE 2. FACTORS AND LEVELS USED FOR FURTHER INVESTIGATION IN 1750 CM^2 ROLLER BOTTLES, (-) LEVEL CORRESPONDS TO BASE CASE THAT CHANGES WERE COMPARED TO.

Factor	(+) level	(-) level
Aeration	Vent cap	Closed cap
Medium Volume	350 ml	600 ml
Cultivation Temperature	35°C	36°C

To ensure that it was possible to measure the 1°C temperature difference and that a larger temperature difference did not accidentally occur, the rollacells, which rotate the roller bottles, were placed in the areas of the incubation chambers that had shown the least disturbance on previous temperature mappings. The incubation chambers were then mapped to ensure an equal temperature distribution over the height of the rollacell and monitored to ensure that the temperature stayed constant over each passage.

4.2.3 MEASUREMENTS

Cell counts

Cells from all experiments were counted using a haemocytometer and the cell viability was determined via trypan blue exclusion assay (Strober, 2001). Viable cells with intact cell membranes exclude the trypan blue dye while non-viable cells absorb the dye. This allows the number of viable and non-viable cells to be counted and the viability of the cell culture to be determined.

For the amplification experiments were there many samples to be processed simultaneously, the cell counting was performed on a Cedex MS20 C (*Innovatis, Germany,* http://www.bioresearchonline.com/product.mvc/Cedex-Automated-Cell-Counter-0001) which also uses a trypan blue exclusion assay. Manual trypan blue exclusion assays were also carried out as a control on the results from the Cedex.

The population doubling level (PDL) was calculated using the results of the cell counts of the number of cells attached to the roller bottle at the end of each passage, as shown in section 8.1.1

Metabolites

Glucose and lactate levels were measured on a Nova Bioprofile 100+ (*Novabiomedical, Waltham, MA*). The Nova Bioprofile 100+ uses amperometric electrodes with immobilized enzymes in their membranes to measure glucose and lactate. These electrodes develop a current proportional to the concentration of the substrate being measured, which can then be interpreted as the glucose and lactate concentrations. pH was measured on a Metrohm pH-meter (*Metrohm, Switzerland*).

The oxygen content of the 850 cm^2 roller bottles was monitored using two different methods. The first method, made use of an external blood gas analyser to analyse medium samples while the second method used sensors immobilized on the wall of the roller bottle. The second allowed daily measurements of both the oxygen in the headspace and the supernatant without opening the roller bottle. Data was also recorded with the first method to enable a comparison to the data from the 1750 cm^2 roller bottles, which was only measured on the blood gas analyser.

In order to better follow the effect of the vent caps, Planar Oxygen Minisensors (*Presens, Germany*) were attached to the inside of the 850 cm^2 roller bottles which allow the measurement the pO_2 in both the medium and the air above the medium in the roller bottles without having to open the roller bottle and thus disturb the system. The sensors were not used on the 1750 cm^2 roller bottles due to time constraints. External pO_2 measurements of the pO_2 in the spent medium were also taken on the ABL5 (*Radiometer, Denmark,* http://www.radiometer.com) at each medium change as a comparison.

pO_2 and pCO_2 were measured on an ABL 5 blood gas analyser (*Radiometer, Denmark,* http://www.radiometer.com). The pCO_2 in the sample is measured on a combined glass and silver/silver chloride reference electrode, immersed in bicarbonate electrode and mounted in a plastic jacket with a silicone membrane. The silicone membrane only allows uncharged molecules of CO_2, O_2 and N_2 to pass through it. Dissolved CO_2 from the sample diffuses through the membrane into the bicarbonate electrolyte, changing the pH of the electrolyte and resulting in a voltage difference over the electrode, which is then converted into a pCO_2 reading, given in mmHg. . The pO_2 is measured on an amperometric electrode that consists of a silver anode and platinum cathode, immersed in a phosphate buffer electrolyte and surrounded by an oxygen permeable membrane. Oxygen from a sample, which diffuses across the membrane into the electrolyte solution, is reduced by the cathode, producing a current that is proportional to the oxygen tension. The pO_2 reading is also given in mmHg.The electrodes are calibrated with mixtures with known pO_2 and pCO_2, which are calculated from Henry's law.

Changes in the glucose, lactate, pH, pCO_2 and pO_2 were calculated by analysing the fresh medium before a medium change and analysing the spent supernatant after a medium change or trypsinisation and then summed over a passage.

Cell state

The apoptosis kit of the Guava EasyCyte™ (*Guava Technologies, Hayward, CA,* www.guavatechnologies.com), which measures the number of viable cells, the number of cells in apoptosis, the number of dead cells and the amount of debris, was used to confirm the viability of cells at the end of a passage.

4.3 BIOREACTOR PROCESS

4.3.1 THE PROCESS

New MBR 40 litre bioreactors with a 28 litre working volume (*New MBR, Switzerland*) were used for the production of the protein in question. The bioreactor process took place in four distinct phases. Phase 1 is the attachment phase: 12-18 x 10^9 cells from the seed train are inoculated into the bioreactor, which already contains microcarriers, and are then left to attach with only agitation, no perfusion. Phase 2 is the growth phase: perfusion and pH control commence and cell growth medium with 5% FBS is used to encourage cell growth. The cell growth phase continues until there are more than 3x10^6 viable cells/ml in the bioreactor. Phase 3 is the rinsing phase: the perfusion medium changes to medium with only 0.05% FBS to remove the FBS used in the growth phase from the bioreactor. Phase 4 is the production phase: the final phase lasts 30 days with harvests every second day, giving a total of 15 harvests per run. The production medium contains only 1% FBS to minimise the amount of protein to be dealt with during downstream processing. Perfusion is measured as volume medium/bioreactor working volume/day (v/v/d). A summary of the controlled parameters for each phase can be seen in Table 3.

TABLE 3. DETAIL OF THE FOUR PHASES OF THE PRODUCTION PROCESS

Parameter	Attachment	Growth	Rinsing	Production
Medium type	Medium + 5% FBS	Medium + 5% FBS	Medium + 0.05% FBS	Medium+1% FBS
Spin filter	65 rpm	65 rpm	70 rpm	70 rpm
Pressure	200 mbar	200 mbar	200 mbar	200 mbar
Impeller speed	45 rpm	45 rpm	50 rpm	50 rpm
Temperature	37.0°C	37.0°C	36.5°C	36.5°C
pO_2	40%	40%	40%	40%
pH	n/a	6.80	6.80	6.70-6.90
Perfusion	n/a	Starts on WD 2 at 1 v/v/day	1.5 v/v/day	Varies from 1-2 v/v/day depending on amount of glucose required by cells - glucose levels must be kept above 1.0 g/L and lactate levels must be kept below 1.5 g/L
Duration	1 Day	5-7 Days, until cells reach >3x10^6 cells/ml	2 Days	30 days

4.3.2 EXPERIMENTAL DESIGN

After a literature survey of the process parameters that it is possible to change and that are likely to have an effect within the prescribed range, pH and perfusion rate were chosen for this investigation.

However, there were several constraints to designing the experiments:

- Very little *a priori* data. Most historic glycosylation data focuses on the final bulks, which are combinations of several harvests, often from more than one bioreactor, making it difficult to determine how the glycosylation varies over time in the process and to what extent it may do so.
- Limited DSP capacity. It was not possible to concentrate each harvest for glycan mapping; some runs were limited to only three harvests out of the fifteen harvests per run, i.e. only three glycosylation data points per run.
- It is unlikely that is will be possible to maintain a constant perfusion rate throughout a run. The perfusion rate usually needs to be increased to keep the glucose concentration above specified levels. Therefore, though the initial perfusion rate at the start of the production phase can be set, it is highly unlikely to stay constant throughout the process.
- There are a limited number of runs due to resource and time constraints.

As the DSP capacity is very limited, there were not enough glycosylation data points per run to analyse each run as time series data. Therefore, the data generated was analysed as individual data points and as average data per run. Both the average data and the individual data points are important as several harvests are usually combined to form a final bulk harvest.

The average data from a run was analysed with ANOVA using the initial conditions of the run, while the data from the individual data points was analysed for their correlation to Z-number (a measurement of glycosylation) with ANCOVA. The initial conditions where then used to design the experiments, the final design was a mixed 2- and 3-level factorial design, with some replicates, as can be seen in Table 4.

TABLE 4. EXPERIMENTAL DESIGN FOR BIOREACTOR EXPERIMENTS BASED ON INITIAL CONDITIONS

Run	pH	Initial perfusion rate (v/v/d)	Method of increasing perfusion rate
Run 1.1	6.7	1.5	As needed
Run 1.2	6.9	1.5	As needed
Run 1.3	6.9	1.0	As needed
Run 2.1	6.8	1.0	As needed
Run 2.2	6.8	1.0	Linearly
Run 2.3	6.7	1.5	As needed
Run 3.1	6.8	1.0	As needed
Run 3.2	6.8	1.0	As needed

4.3.3 MEASUREMENTS TAKEN

Cell counts

Cells in suspension and on microcarriers were counted daily. Cells on microcarriers were first detached from microcarriers using trypsin before being counted. Cells were counted manually using a haemocytometer and the same trypan blue exclusion assay as for the roller bottles. Viable cells with intact cell membranes exclude the trypan blue dye while non-viable cells absorb the dye. This allows the number of viable and non-viable cells to be counted and the viability of the cell culture can be determined.

Metabolites

The Nova Bioprofile100+ (*Novabiomedical, Waltham MA,* http://www.novabiomedical.com) was used to measure the glucose, lactate, glutamine, glutamate concentrations with amperometric electrodes, which develop a current proportional to the concentration of the substrate being measured and ammonium, sodium and potassium concentrations were measured with potentiometric electrodes that develop a voltage proportional to the concentration of the ion being measured.

A pH meter (*Metrohm, Switzerland*) was used to check the pH of the bioreactor externally.

pO_2 and pCO_2 were measured on an ABL 5 blood gas analyser (*Radiometer, Denmark,* http://www.radiometer.com). The pCO_2 in the sample is measured on a combined glass and silver/silver chloride reference electrode, immersed in bicarbonate electrode and mounted in a plastic jacket with a silicone membrane. The silicone membrane only allows uncharged molecules of CO_2, O_2 and N_2 to pass through it. Dissolved CO_2 from the sample diffuses through the membrane into the bicarbonate electrolyte, changing the pH of the electrolyte and resulting in a voltage difference over the electrode, which is then converted into a pCO_2 reading. The pO_2 is measured on an amperometric electrode that consists of a silver anode and platinum cathode, immersed in a phosphate buffer electrolyte and surrounded by an oxygen permeable membrane. Oxygen from a sample, which diffuses across the membrane into the electrolyte solution, is reduced by the cathode, producing a current that is proportional to the oxygen tension. Both results are gicven in mmHg. The electrodes are calibrated with mixtures with known pO_2 and pCO_2, which are calculated from Henry's law.

NaOH and antifoam consumption as well as perfusion were measured by weight.

4.4 GLYCOSYLATION ANALYSIS

The glycosylation was characterised by determining the glycan charge profile of each harvest – i.e. the amount of neutral, mono-sialylated, di-sialylated, tri-sialylated and tetra-sialylated glycan species. The percentage of each species was then used to calculate the Z-number for each harvest. See appendix 8.1 for details of Z-number calculation.

Usually the glycosylation is only monitored on the final bulks (combination of several harvests) which have already been through several downstream processing (DSP) steps including several filtration and chromatography steps. However, the DSP processing lowers the Z-number, especially during the chromatography steps and it is uncertain whether this is a predictable effect. In order to avoid any obscurity due to the effect of DSP, it was decided to analyse the glycan charge profile before the first chromatography step. Glycans from concentrated harvests were enzymatically detached from proteins using PNGase, then labelled with 2-aminobenzamide before HPLC analysis to determine their respective charges (Fernandes, 2006).

5 RESULTS AND DISCUSSION

The aim of this study was to investigate the effect of the medium volume, cultivation temperature, seeding density, medium feed temperature and aeration on the cell growth in the seed train and of the pH and perfusion rate on the glycosylation of the product during production. The results of the experiments described in the previous section will be discussed below, first for the seed train and then for the production phase.

5.1 SEED TRAIN RESULTS

To investigate the effect of the seeding density, medium volume, aeration, cultivation temperature and medium feed temperature on the cell growth and final PDL in the seed train, experiments were performed in 850 cm^2 and 1750 cm^2 roller bottles. The results of these experiments were analysed using Statistica™'s Design of Experiments package. Analysis of variance (ANOVA) was performed on the results using a 95% confidence level. Neither 3rd-order not 2nd-order interactions were found to have a significant effect on the cell culture. The results of the influence of the main factors are presented in the following sections.

5.1.1 CELL GROWTH AND PDL

The number of cells produced by the seed train and the final PDL are important characteristics of a seed train as they are two of the three parameters (along with the viability) used to determine the success of a seed train and whether those cells will be used for production.

An ANOVA was performed on the PDL after one passage in the experiments described in section 4.2. The seeding density proved to be the only variable that had a significant effect on the PDL, as seen in Figure 6 and Figure 7.

Regardless of the initial seeding density, there is no significant difference in the final number of cells produced, as can be seen in Figure 6. The same trend is also observed in the larger 1750 cm^2 roller bottles.

Cell growth was followed by observation through a microscope during each passage. Initially the cells were flat and spread out on the surface of the roller bottles. By working day five, the same observation could be made for all the roller bottles, regardless of cultivation conditions: cells were round, packed very tightly together, appeared to pop-up off the surface of the roller bottle and had completely covered the entire surface of the roller bottle. In other words, the cells had attained confluence and had no further space in which to grow.

From microscopic observation, it appears that the cells reach confluence by working day five, regardless of the seeding density. It appears that the growth of the cells used in this process is inhibited once they reach confluence. The results seen here correspond to the results achieved by Wu et al. (2005) with MRC-5 cells where a lower seeding density lead to a higher final PDL as cell growth was inhibited once the cells reached confluence.

Contact inhibition of cell growth is a normal characteristic of mammalian cells. That it was not observed in the internal study (105923, 2006), which was performed with CHO cells, indicates that researchers harvested the cells too soon, before inhibition could be observed, or that the cells were modified to remove this characteristic. These experiments confirm that the growth of the specific CHO cells used in this process is inhibited when the cells reach confluence.

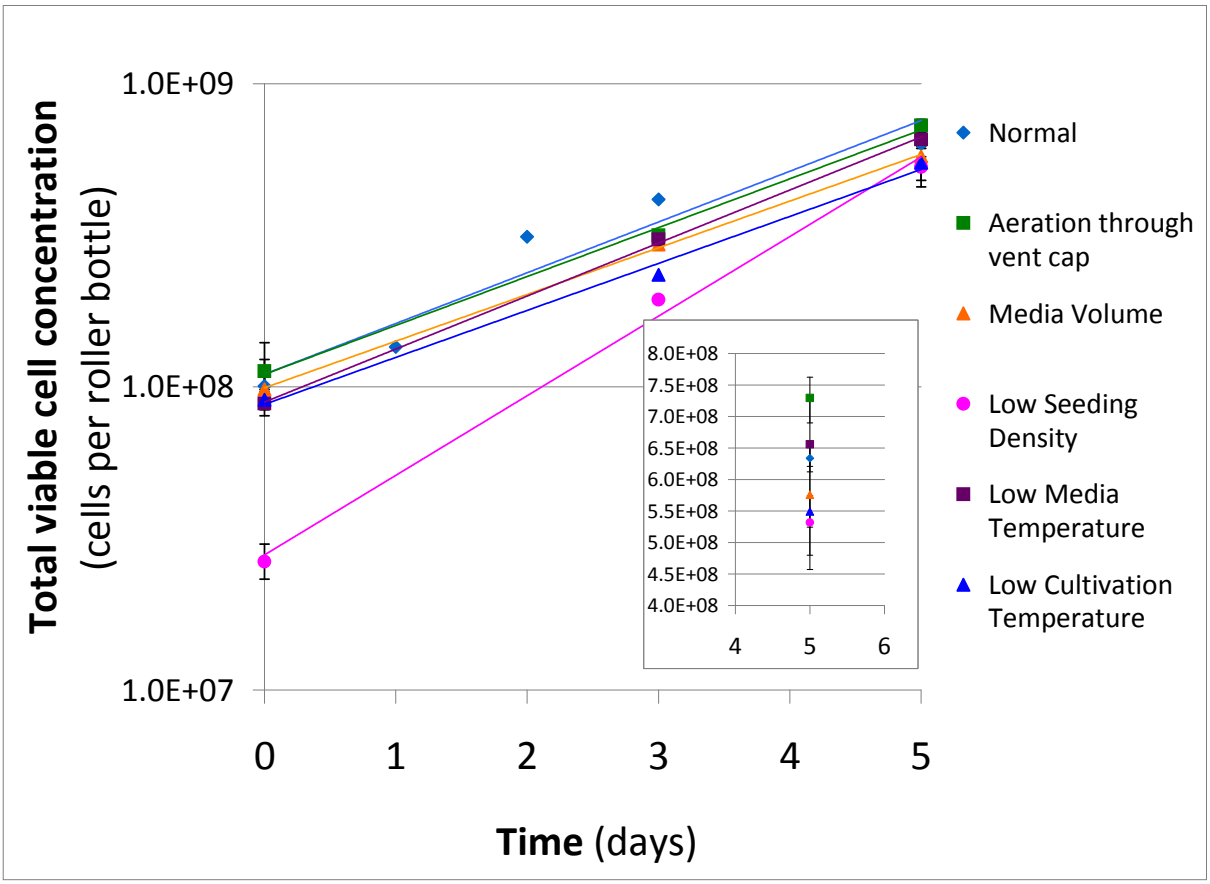

FIGURE 6. GROWTH CURVES OF VIABLE CELLS IN 850 CM^2 ROLLER BOTTLES (ERROR BARS REPRESENT THE DATA RANGE AFTER THREE REPLICATES, THOUGH THE DATA ON DAYS 1, 2 AND 3 DO NOT HAVE REPLICATES). THE GROWTH CURVES REPRESENT THE BASE CASE USED AS A CONTROL (NORMAL) AND THE CHANGES MADE TO THE BASE CASE: THE ADDITION OF A VENT CAP FOR AERATION, THE USE OF A LOW MEDIUM VOLUME, LOW SEEDING DENSITY, MEDIUM AT A LOW TEMPERATURE FOR MEDIUM EXCHANGE (REPLACE SPENT MEDIUM WITH FRESH MEDIUM) AND A LOW CULTIVATION TEMPERATURE. THE INSERT GIVES A BETTER VIEW OF THE DATA POINTS ON DAY 5.

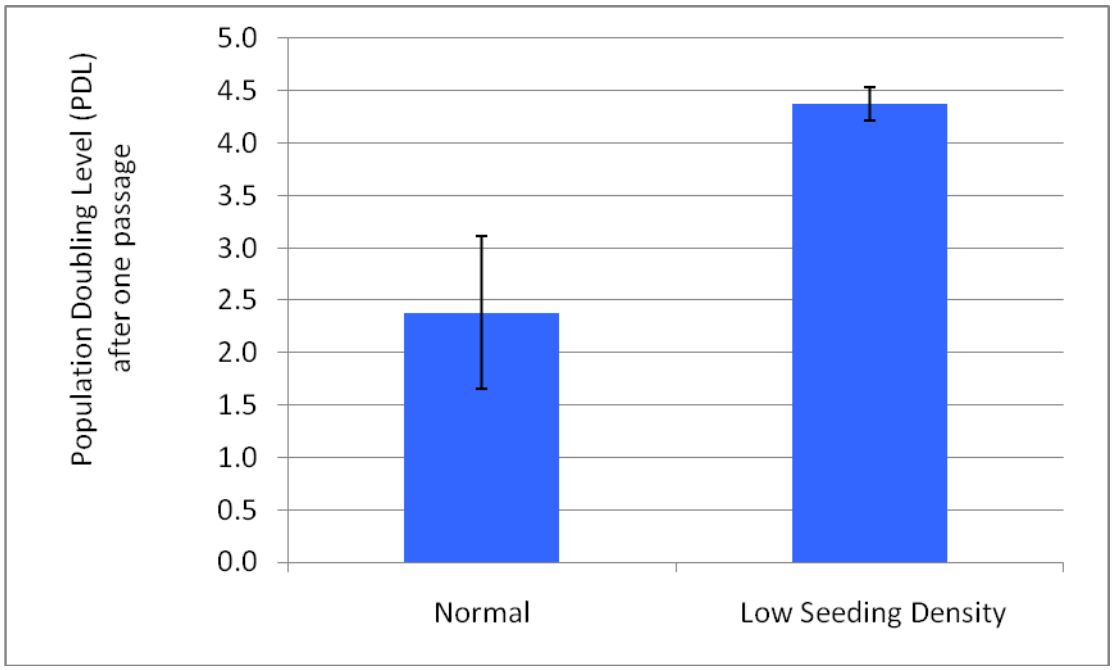

FIGURE 7. POPULATION DOUBLING LEVEL AFTER ONE PASSAGE IN 850 CM^2 ROLLER BOTTLES WITH A ROLLER BOTTLE CULTURED AT NORMAL CONDITIONS (AS A CONTROL) AND A ROLLER BOTTLE WITH A LOW SEEDING DENSITY (ERROR BARS REPRESENT THE DATA RANGE AFTER 6 REPLICATES).

The PDL for each passage is calculated from the number of viable cells used for inoculation and the total number of cells harvested at the end of the passage. As can be seen from Figure 7, using a lower seeding density significantly increased the PDL at the end of a passage; as all the roller bottles yielded similar numbers of cells at the end of the passage the size of the increase is determined by the seeding density used for inoculation.

The final PDL of a seed train is calculated by measuring the PDL after each passage and then adding it up over the five passages that make up the seed train. As the final number of cells harvested from the roller bottles appears to be the same regardless of the growth conditions and seeding density, it ought to be possible to influence the final PDL by influencing the seeding density of each passage. The seeding density of the first two passages is predetermined. The first passage in T-flasks is inoculated using all the cells from the ampoule from the cell bank, while the second passage in 850 cm^2 roller bottles is inoculated using all the cells obtained from the T-flasks, that leaves the last three passages in the 1750 cm^2 roller bottles where the seeding density can be adjusted.

The seeding density of the 1750 cm^2 roller bottles is 150-200 x 10^6 cells. The range of the PDL at the end of the first two passages can be determined from historic data as 66-68, and the range of the number of cells harvested from the roller bottles is 1500-1800 x 10^6 cells. From this data, it is possible to theoretically predict the final PDL at the end of the seed train, as seen in Figure 8, with the minimum and maximum points based on having all three the final passages inoculated with 150 x 10^6 and 200 x 10^6 cells, respectively. See Appendix 8.1 for PDL calculations.

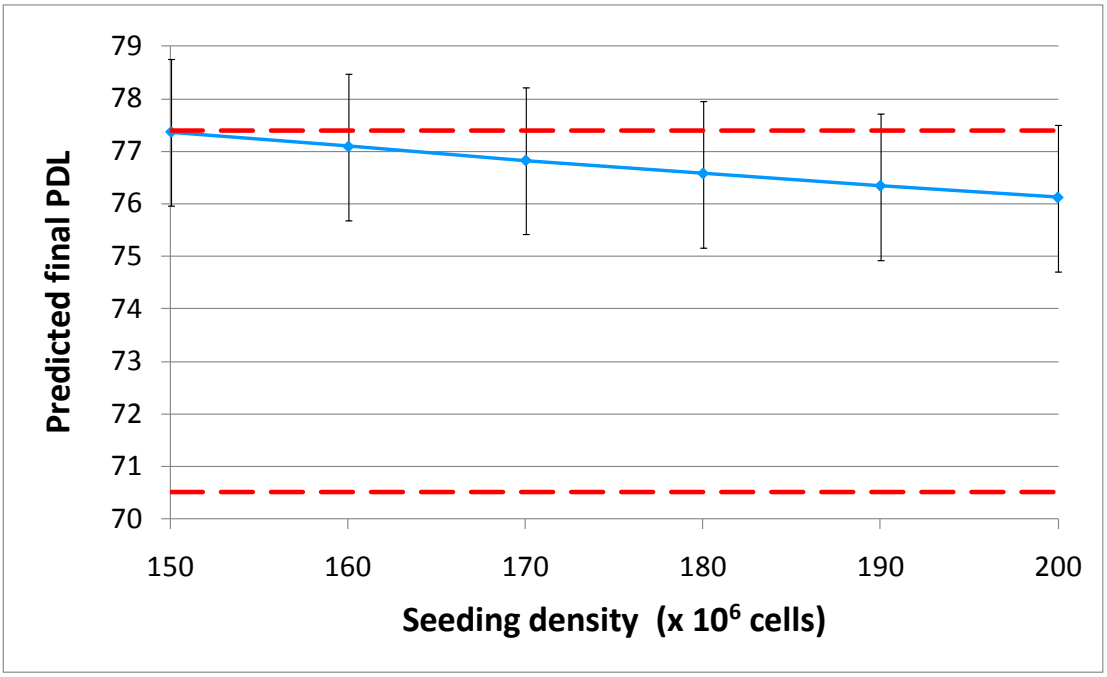

FIGURE 8. PREDICTED FINAL PDL BASED ON INOCULATION DENSITY USED IN LAST THREE PASSAGES, WITH RED DASHED LINES INDICATING THE DESIRED RANGE OF THE FINAL PDL (ERROR BARS INDICATE THE RANGE OF PREDICTION BASED ON THE RANGE OF PDL AFTER FIRST TWO PASSAGES AND THE RANGE OF THE NUMBER OF CELLS HARVESTED).

From Figure 8 it is clear that without careful cell counting it is possible to exceed the desired ranges of the final PDL and that a higher seeding density leads to a lower final PDL. Interestingly, historic data indicates that operators appear to usually use a higher seeding density, as seen in Figure 9. Therefore, despite being the only controlled process variable of those tested that had a significant influence the cell growth and final PDL, the seeding density does not appear to have been the cause of the apparent increase in the final PDL.

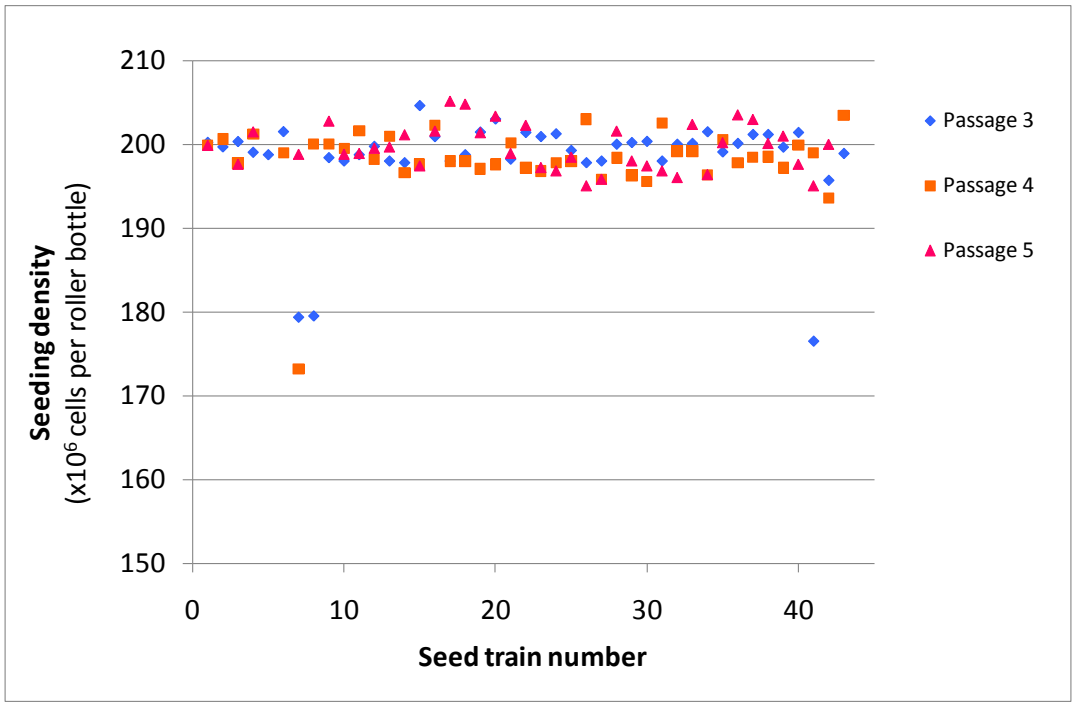

FIGURE 9. HISTORIC DATA OF SEEDING DENSITY USED TO INOCULATE THE LAST THREE PASSAGES OF THE SEED TRAIN, SEEDING DENSITY SHOULD BE IN THE RANGE OF 150-200 X 10^6 CELLS PER ROLLER BOTTLE. PASSAGE 3, 4 AND 5 INDICATE THE THREE FINAL PASSAGES OF THE SEED TRAIN THAT ARE PERFORMED IN 1750 CM^2 ROLLER BOTTLES.

Figure 10 shows the final PDL at the end of each seed train used in each production campaign. Though the final PDL appears to increase with each new campaign, the final PDL within each campaign remains relatively constant. Campaigns are performed every 1-4 years, and usually there are different operators that manage the seed train for each campaign. A previous investigation eliminated differences in the raw materials and most uncontrolled process variables used in each campaign as the cause of this apparent increase which leaves only the controlled process variables of which only the seeding density significantly affects the final PDL.

Historic data, seen in Figure 9, shows that a seeding density of approximately 200 x 10^6 cells was used in each of the final three passages of each seed train; theoretically this should give a final PDL of approximately 75-78 (as seen in Figure 8). The apparent increase in final PDL falls within this range from 75-77, as seen in Figure 10. As the seeding density appears to be approximately constant, the differences in final PDL are probably due to variation in the cell yield at the end of the three final passages and the PDL after the first two passages. Both these sources of variation could be due to true variation in cell yields or to variation based on differences in cell counting between operators.

In summary, the final PDL remains relatively constant within each campaign (as seen in Figure 10) and no significant difference could be found in the final cell yields at the end of each passage in these experiments (see Figure 6), which comprised four seed trains where operator cell counts were verified with an electronic cell counting machine (Cedex MS20 C). Therefore, it is concluded that the apparent increase in final PDL is more likely due to variation in cell counting between operators in different campaigns than a true increase in the final PDL.

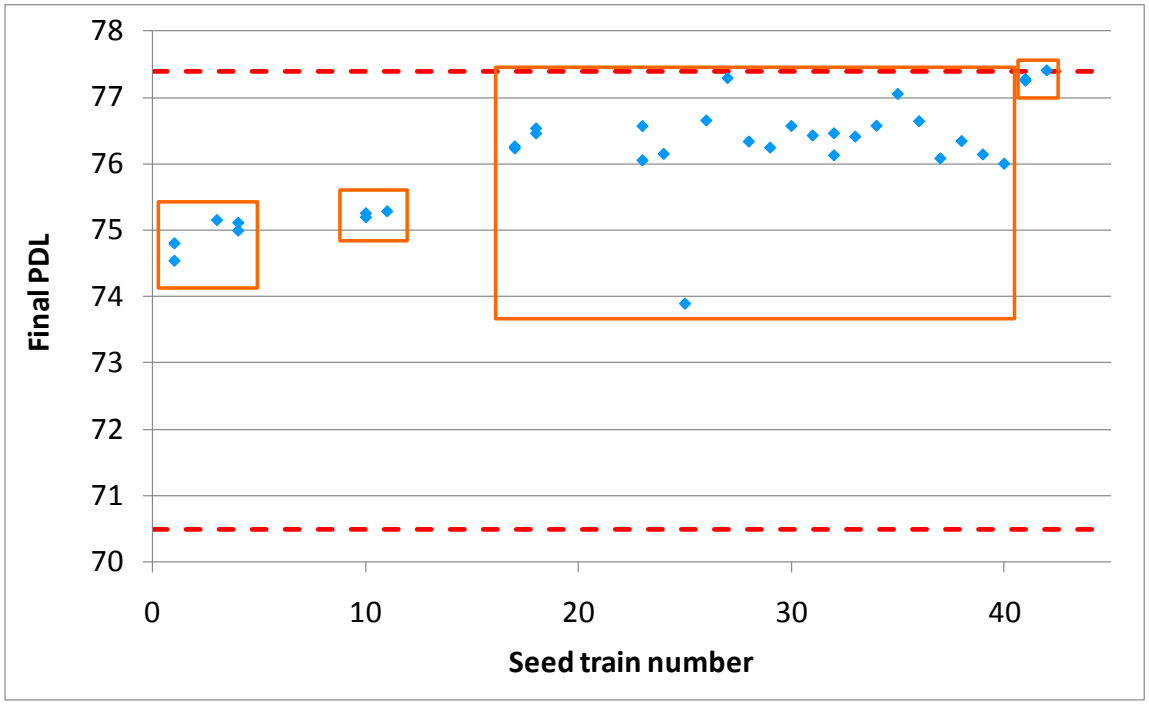

FIGURE 10. HISTORIC DATA OF FINAL PDL AT THE END OF EACH SEED TRAIN, ORANGE BLOCKS INDICATE DIFFERENT PRODUCTION CAMPAIGNS AND THE DASHED RED LINES INDICATE THE FINAL PDL SPECIFICATION LIMITS.

5.1.2 CELL VIABILITY

The cell viability is the third criterion by which a seed train is judged; the viability of the cells at the end of the seed train should be above 80%. Historically this has not been a problem, but to further examine the state of the cells in the roller bottles at the end of a passage under various cultivation conditions apoptosis tests were performed on the cells to determine the number of viable cells, the number of apoptotic cells and the number of dead cells.

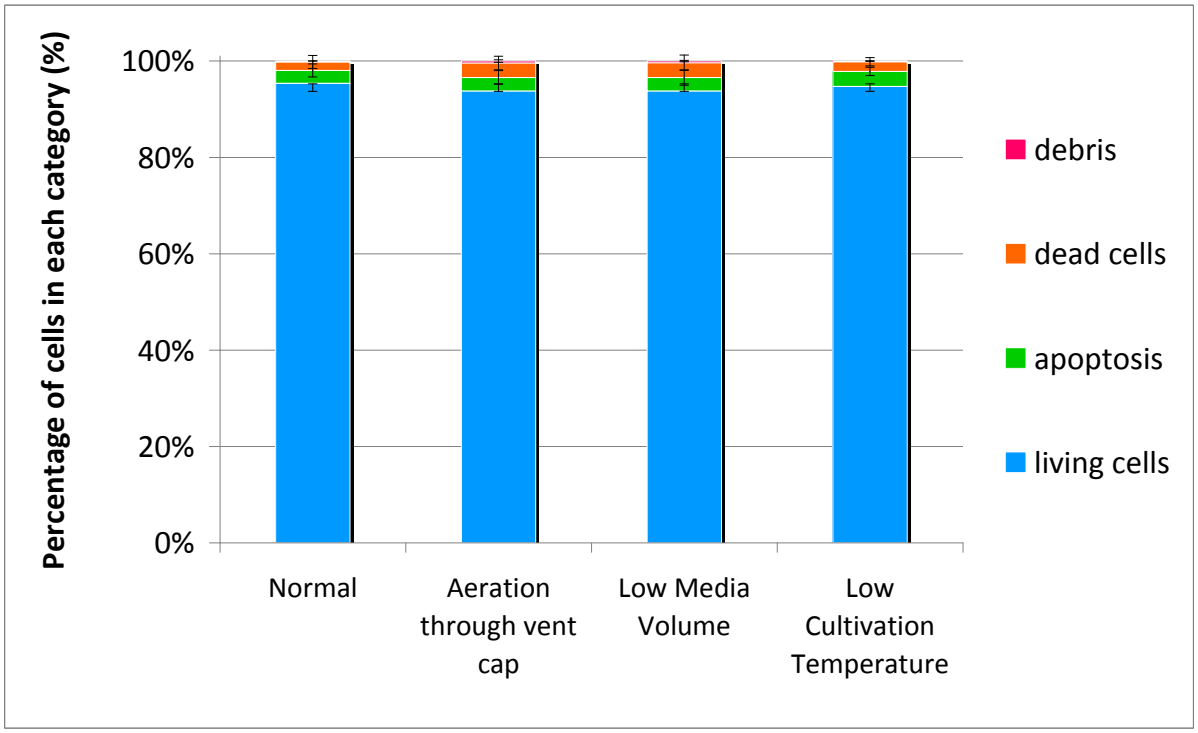

FIGURE 11. PERCENTAGE OF VIABLE, APOPTOTIC AND DEAD CELLS AFTER A PASSAGE UNDER VARIOUS CULTIVATION CONDITIONS IN 1750 CM^2 ROLLER BOTTLES (ERROR BARS REPRESENT DATA RANGE AFTER THREE REPLICATES). THE CONDITIONS SHOWN ARE THE BASE CASE (NORMAL) AND THE CHANGES MADE TO THE BASE CASE: AERATION THROUGH A VENT CAP, LOW MEDIUM VOLUME IN THE ROLLER BOTTLE AND LOW CULTIVATION TEMPERATURE.

All of the cultivation conditions used in these experiments lead to a viability of greater than 90% in both the 850 cm^2 and the 1750 cm^2 roller bottles, with no significant difference between the number of cells in apoptosis, as can be seen in Figure 11. None of the controlled process parameters tested appears to influence the viability of the cells. Historically the final viability of the cells from a seed train has also been above 90%, so the data produced in these experiments corresponds to the historic data.

5.1.3 CELLS IN SUSPENSION

An ANOVA performed on the total cumulative cells in suspension showed that low medium volume was the only factor to make a significant difference to the total number of cells in suspension; the effect was seen in both the 850 cm^2 and 1750 cm^2 roller bottles. The trends of the number of cells in suspension, as seen in Figure 12, show that both roller bottles with a low medium volume and the roller bottles with a low seeding density followed different trends to the norm.

The number of cells in suspension generated by the roller bottles with a low seeding density increased as the number of cells in the roller bottle increased, i.e. parallel to the growth curve seen in Figure 6, to finally yield the same total number of cells in suspension as the roller bottles inoculated with a normal seeding density. Unlike roller bottles with a low seeding density, roller bottles with a low medium volume start with the same number of cells as normal and also have a similar number of cells in suspension when the medium is changed for the first time on working day two. These cells are likely the dead cells or excess cells that did not attach to the roller bottle walls. However, after working day two, very few cells in suspension are generated.

From microscopic observation of the cells, it was seen that as the cells grew and became confluent, they tended to round up. These rounded-up cells could then be detached from the surface of the roller bottle by the drag force of the medium moving down the side of the roller bottle as it is rotated. A smaller medium volume would mean that the force on the cells, the size of which is mainly determined by the weight of the medium and the rotational speed of the roller bottle, is less, as the rotational speed of the roller bottle was kept constant for all conditions.

Experiments and simulations of the mixing in a roller bottle by Unger *et al.* (2000) provide support for this theory. They show that the highest liquid velocity is at the surface of the roller bottles, moving tangentially along the surface in the opposite direction to the rotation of the roller bottle, as would be necessary for the volume to affect the number of cells in suspension.

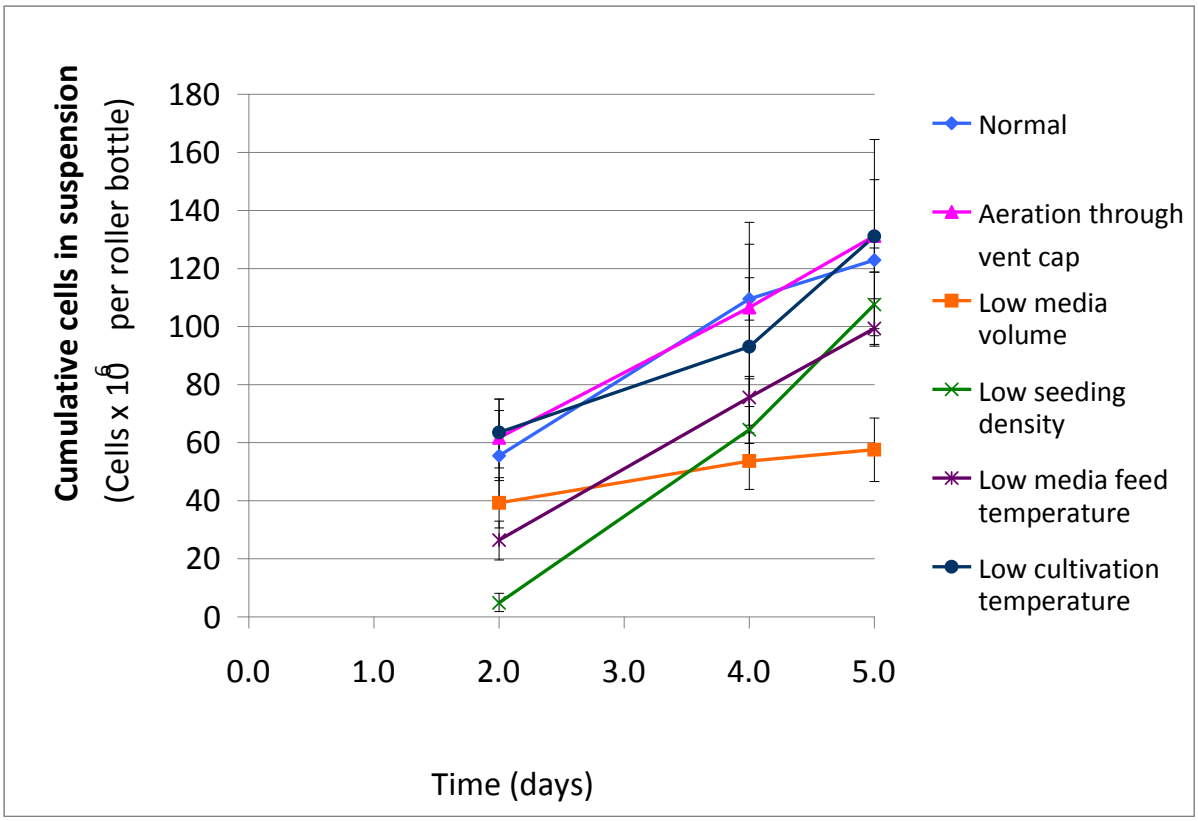

FIGURE 12. CUMULATIVE CELLS IN SUSPENSION IN 850 CM² ROLLER BOTTLES (ERROR BARS REPRESENT THE DATA RANGE AFTER SIX REPLICATES). THE DATA REPRESENTS THE BASE CASE (NORMAL) AND THE CHANGES MADE TO THE BASE CASE: AERATION THROUGH A VENT CAP (AERATION), LOW MEDIUM VOLUME, LOW SEEDING DENSITY, LOW MEDIUM TEMPERATURE AT MEDIUM ADDITION AND A LOW CULTIVATION TEMPERATURE.

Wu et al. (2005) found that increasing the medium volume in MRC-5 cells lead to a higher final PDL. They suggested that this was due to either an increase in the availability of nutrients or to the decrease in oxygen tension. This was not the case in this process and though there is a difference in the number of cells in suspension, this does not make a significant difference to the cell growth or the final PDL. It is possible that the change in availability of nutrients was not large enough to observe this difference. Ryan et al. (1975) found that doubling the cell culture volume yielded a two-fold increase in the cell yield. In these experiments, the medium volume was almost doubled, and no significant increase in cell yield was observed. It would appear that with the current medium composition there are sufficient nutrients available for the cells regardless of the volume used. No other authors had reported the similar effects of the medium volume on the number of cells in suspension. The lower medium volume might lead to a great oxygen availability as there is more headspace in the roller bottles, but during these experiments it did not have a significant effect (see 5.1.5).

5.1.4 GLUCOSE AND LACTATE CONSUMPTION

Glucose is the main source of carbon for the cells and is usually metabolised via glycolysis to pyruvate and then goes on the become lactate or to enter the TCA cycle. An ANOVA of the total cumulative glucose consumption over a passage showed that the aeration, medium volume, seeding density and cultivation temperature all had a significant effect in the 850 cm^2 roller bottles, as shown in Figure 13. Similar trends were found for the 1750 cm^2 roller bottles though the smaller temperature difference (35-36°C) tested in the 1750 cm^2 roller bottles did not have a significant effect. The trends in lactate production reflect the trends in glucose consumption in both 850 cm^2 and 1750 cm^2 roller bottles.

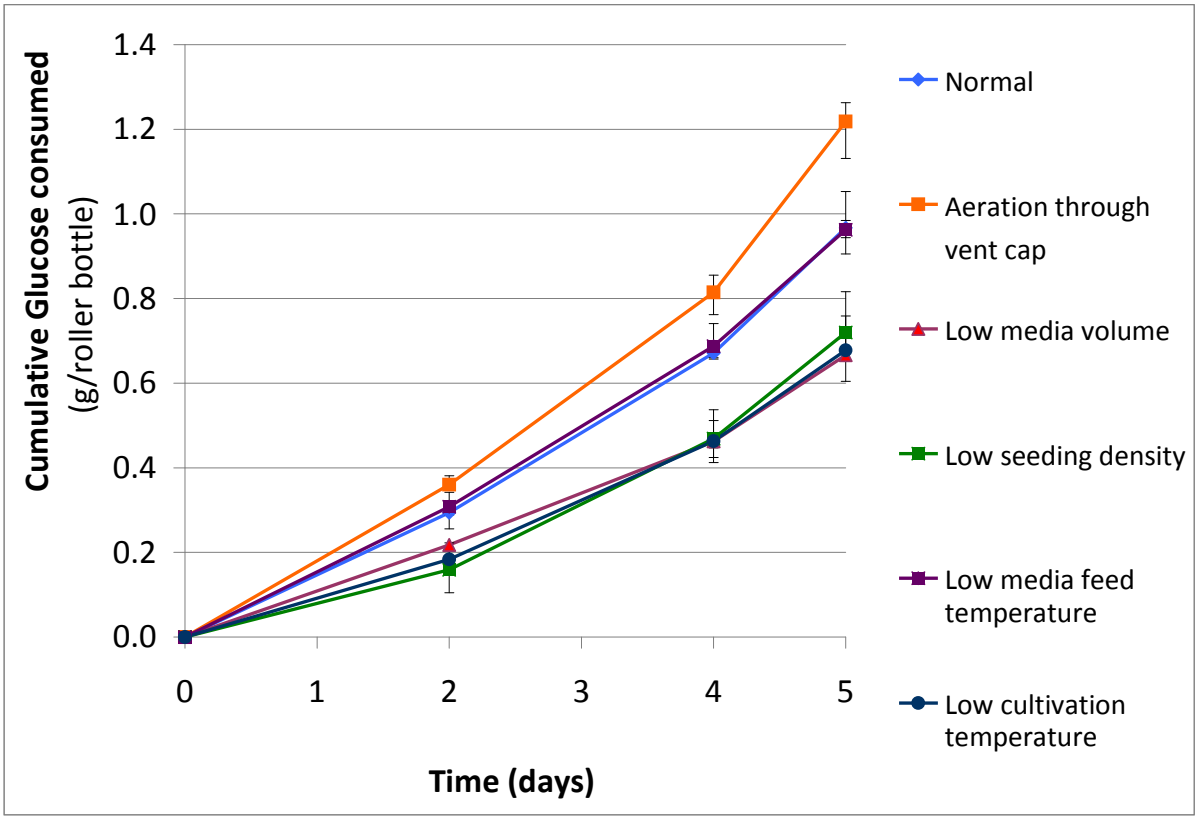

FIGURE 13. CUMULATIVE GLUCOSE CONSUMPTION IN 850 CM^2 ROLLER BOTTLES (ERROR BARS REPRESENT DATA RANGE AFTER SIX REPLICATES). THE DATA REPRESENTS THE BASE CASE (NORMAL) AND THE CHANGES MADE TO THE BASE CASE: AERATION THROUGH A VENT CAP (AERATION), LOW MEDIUM VOLUME, LOW SEEDING DENSITY, LOW MEDIUM TEMPERATURE AT MEDIUM ADDITION AND A LOW CULTIVATION TEMPERATURE.

The roller bottles with a low seeding density had a lower total glucose consumption compared to normal. The most probable cause is the low inoculation; because they started with fewer cells, less glucose than normal was initially consumed.

The roller bottles with a lower cultivation temperature also consumed less glucose than normal. The decrease in the total glucose consumption in roller bottles cultivated at 35°C instead of at 37°C is possibly due to the metabolism of the cells slowing down. The slower cell growth that often accompanies slower metabolism is not statistically significant, but these roller bottles did have the slowest growth curve and lowest number of cells harvested, as can be seen in Figure 6. Lowering the cultivation temperature of cells is a common technique used to control cell cultures, and the differences in metabolism observed here correspond to differences observed by Rössler et al. (1996) and Tsao et al. (1992); however, there is no clear explanation for why the statistically significant decrease in glucose consumption is not reflected in the cell growth.

The decrease in the total glucose consumption in roller bottles cultivated at a lower medium volume is probably due to the decreased contact time between the cells and the medium. At the same rotational speed, as is the case here, a roller bottle with less medium that therefore has a smaller contact surface with the cells, will have less contact time with the cells than a roller bottle with a higher medium volume.

The increased glucose consumption in the aerated roller bottles might be due to the increased availability of oxygen but this is uncertain, as no significant increase in cell growth has been observed.

5.1.5 PARTIAL PRESSURE OF OXYGEN AND CARBON DIOXIDE

Oxygen

The oxygen content of the 850 cm^2 roller bottles was monitored using two different methods. The first method, made use of an external blood gas analyser to analyse medium samples while the second method used sensors immobilized on the wall of the roller bottle. The second allowed daily measurements of both the oxygen in the headspace and the supernatant without opening the roller bottle. Data was also recorded with the first method to enable a comparison to the data from the 1750 cm^2 roller bottles.

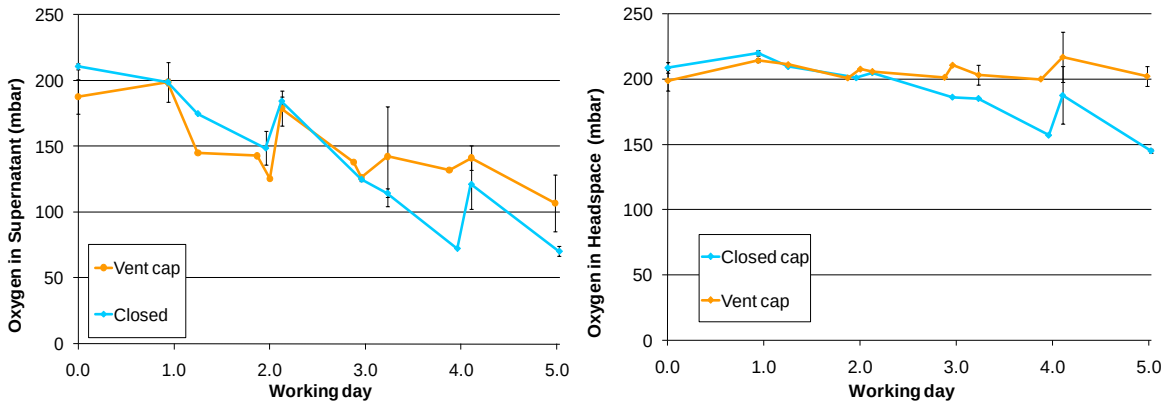

FIGURE 14. OXYGEN CONTENT IN SUPERNATANT AND HEADSPACE OF ROLLER BOTTLES, WITH AND WITHOUT VENT CAPS, AS MEASURED WITH SENSORS IMMOBILISED ONTO THE INTERNAL WALL OF THE ROLLER BOTTLE IN 850 CM^2 ROLLER BOTTLES (ERROR BARS REPRESENT THE RANGE OF THE DATA AFTER THREE REPLICATES)

Figure 14 shows the trend of oxygen partial pressure in the supernatant and headspace of normal roller bottles and roller bottles with a vent cap, as measured by the immobilized sensors. The significant changes on working day 2 and working day 4 are caused by medium changes, as the fresh medium has a higher oxygen content than the spent supernatant. Unfortunately, as the aeration through the vent cap is passive, it is not possible to measure it directly, and the oxygen consumption by a roller bottle with a vent cap can only be approximated. However, the vent caps do seem to allow an increase in the amount of oxygen in the roller bottle. This is especially noticeable toward the end of the passage when there are more cells.

An ANOVA on the change in oxygen content in the supernatant of the roller bottles per passage indicated that the medium feed temperature was one of the factors that significantly influenced oxygen utilisation. However, when the oxygen content trends were compared it appeared that the oxygen content of the cold medium was only higher for the first few hours until it warmed up, and thereafter it followed the normal trend. It was decided to exclude feed temperature from the ANOVA of the change in oxygen content.

The significant factors in 850 cm^2 roller bottles then proved to be cultivation temperature and aeration, as shown in Figure 15. However, in 1750 cm^2 roller bottles none of the factors investigated appeared to significantly influence the oxygen content of the supernatant.

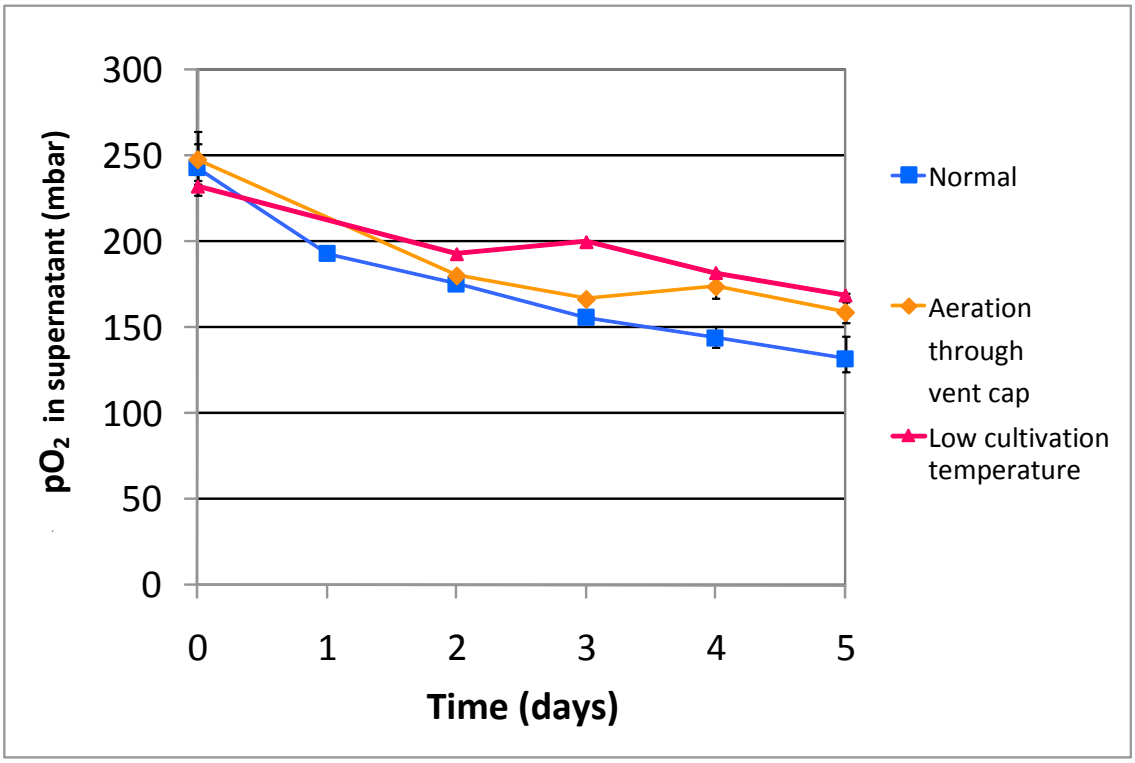

FIGURE 15. OXYGEN PARTIAL PRESSURE IN THE SUPERNATANT OF THE 850 CM^2 ROLLER BOTTLES AS MEASURED WITH AN ABL5 BLOOD GAS ANALYSER (ERROR BARS REPRESENT DATA RANGE AFTER SIX REPLICATES).

The increased oxygen partial pressure seen at 35°C compared to 37°C could be another indication of decreased metabolic rates at a lower temperature to match the decrease in glucose consumption seen Figure 13, but as no significant difference in cell growth was observed this could equally be due to the difference in the solubility of oxygen. Oxygen is more slightly soluble in water at 35°C than at 37°C.

Oxygen solubility in seawater at 1 bar and 35°C is 5.6 mg/L compared to 5.3 mg/L at 40°C (Perry and Green, 1997). Though cell culture medium does not have the same salinity as seawater, this is the closest published comparison it was possible to find.

The difference in the partial pressure of oxygen at the end of a passage at 35°C and at 37°C is ~40 mbar. Given the relatively low difference in solubility and that the only significant indications of a slower metabolism is a reduction in glucose consumption and lactate production, it is likely that the difference in partial oxygen pressure is as a result of a combination of these factors.

The vent cap is effective in increasing the oxygen content of the medium in the smaller 850 cm^2 roller bottles but not in the larger 1750 cm^2 roller bottles. Both roller bottle sizes have the same diameter, but the 1750 cm^2 roller bottle is twice as long as the 850 cm^2 roller bottle. This means that the medium surface area of the larger roller bottle in contact with the air is twice as large as the medium surface area of the smaller roller bottle and that fresh air that comes in through the vent cap has to move twice as far along the length of the roller bottle. This increase in length is probably why there is no significant difference in the oxygen content of the supernatant in the larger roller bottles.

There have been no studies on the mixing of air in roller bottles; however, the mixing of liquid in a roller bottle has been thoroughly studied by Unger *et al.* (2000). Unger *et al.* (2000) found that mixing in the liquid in a roller bottle was far more effective in a radial direction than axial direction (along the length of a roller bottle). With the vent cap on one end of the roller bottle, a similar phenomenon would probably be observed in the distribution of oxygen in the supernatant. With significantly increased oxygen in the part of the medium closer to the vent cap (as seen in the smaller roller bottle) and decreased mixing as the length of the bottle increases so that there is no overall significant difference in the oxygen content of the medium in the larger roller bottle.

Unger *et al.* (2000) found that increasing the rotation speed did not greatly increase the mixing along the length of the roller bottle, mixing only increased in the radial direction, i.e. in the direction of the rotation and instead proposed a new rotation system for roller bottles that also rotates in the axial direction allowing for better overall mixing. In addition, the air filter is relatively small compared to the size of the roller bottle cap and might not have a sufficient capacity for the larger roller bottles.

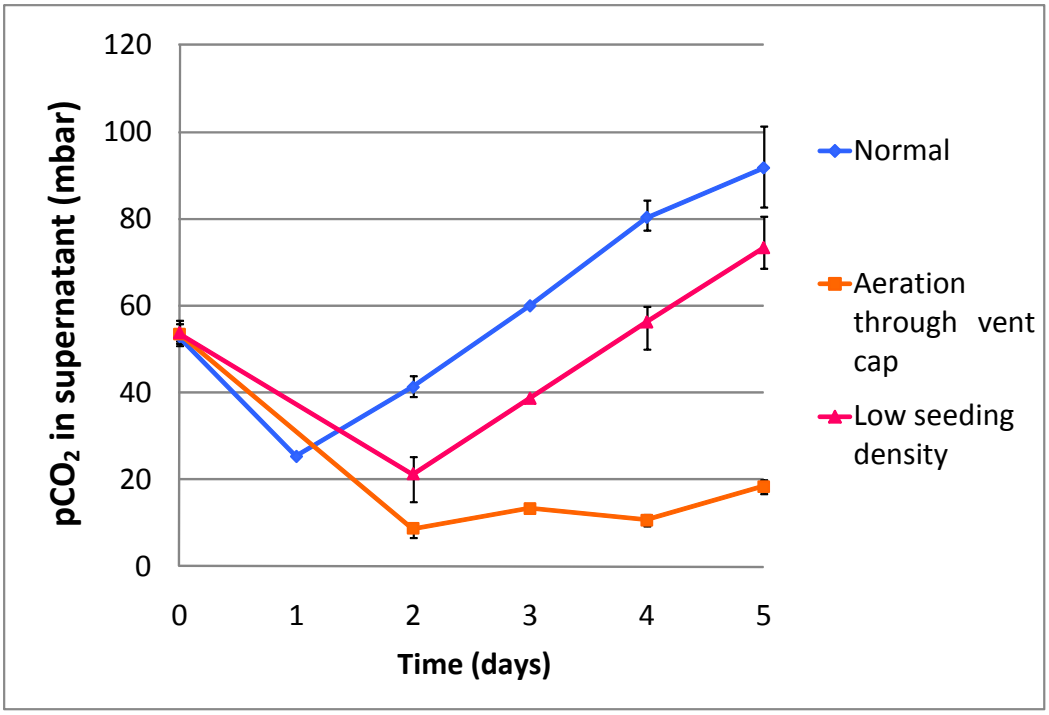

FIGURE 16. PARTIAL PRESSURE OF CARBON DIOXIDE IN SUPERNATANT OF THE 850 CM² ROLLER BOTTLES (ERROR BARS REPRESENT THE RANGE OF DATA AFTER SIX REPLICATES)

The carbon dioxide content of the supernatant was measured using an external blood gas analyser. An ANOVA on the change in the carbon dioxide content of the supernatant revealed that the seeding density and aeration had a significant effect, as can be seen in Figure 16.

Two distinct trends can be seen in the carbon dioxide content of the supernatant from Figure 16. The carbon dioxide content in the supernatant of the roller bottles with a low seeding density appears to follow the normal trend, lagging slightly because the roller bottle initially started with fewer cells. The carbon dioxide in the roller bottles with a vent cap stayed low throughout the passage, with the final difference larger than that of the partial pressure of oxygen in the supernatant between the normal roller bottles and the roller bottles with vent caps. The difference between the final oxygen content and the final carbon dioxide content is probably because carbon dioxide is more stable in the medium than oxygen (carbon dioxide is approximately 30 times more soluble than oxygen in water at 36°C).

Vent caps do make a difference to the oxygen and carbon dioxide content of the supernatant, especially in the smaller roller bottles where a slight increase in metabolism is also observed (increased glucose consumption). However, no significant difference is observed in the cell growth, final PDL or cell viability. This suggests that Wu et al. (2005)'s theory that the decrease in oxygen tension at higher medium volumes lead to a higher PDL when using MRC-5 cells is not applicable to the CHO cells used in this process.

5.1.6 pH

The pH of the medium in the roller bottles changes over the course of a passage (even with medium changes) as the number of cells increases and more waste products are produced. As the optimum pH for growth of the cell line is around pH 7 (the pH of the medium), one would ideally like to keep the change as small as possible. After an ANOVA of the change in pH over a passage in 850 cm^2 roller bottles it was found that aeration, seeding density and a lower cultivation temperature all appeared to cause smaller changes in pH than the normal, as can be seen in Figure 17. None of the factors tested in 1750 cm^2 roller bottles appeared to make a difference.

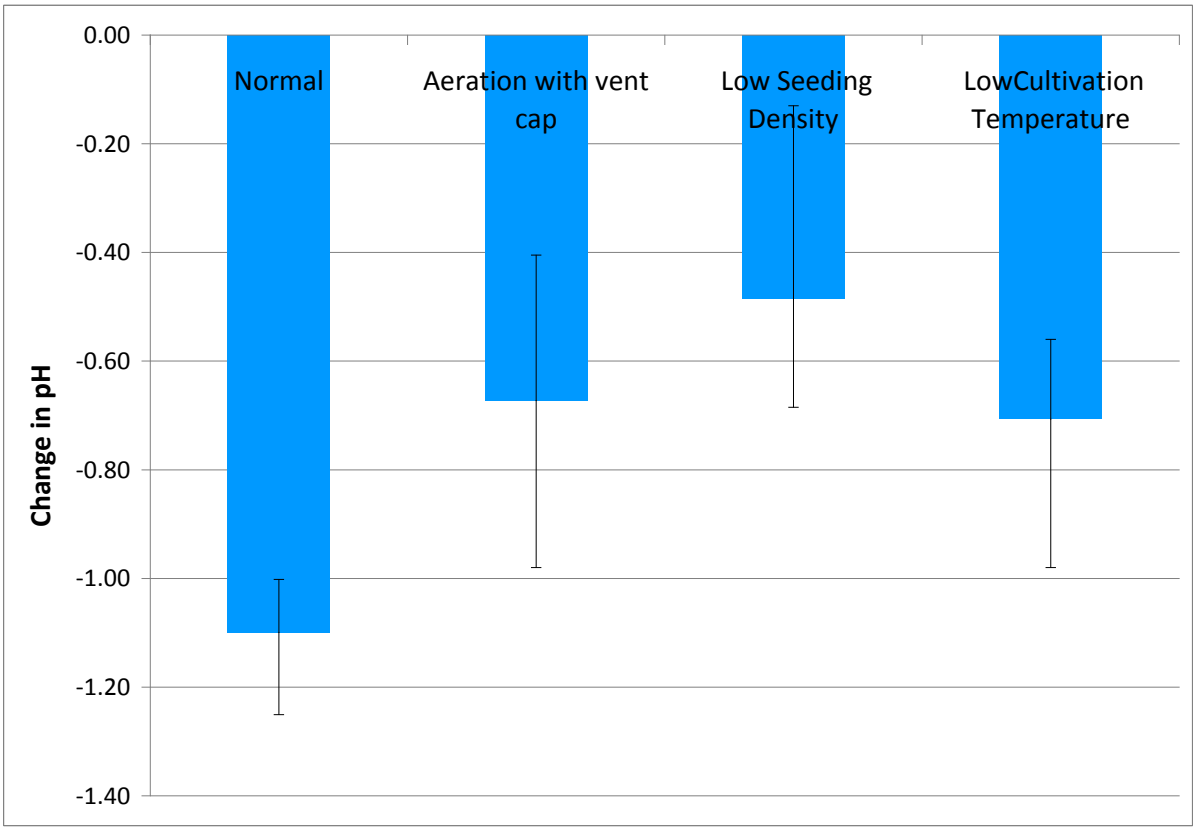

FIGURE 17. CHANGE IN PH OF THE SUPERNATANT IN 850 CM^2 ROLLER BOTTLES OVER A PASSAGE (ERROR BARS REPRESENT THE RANGE OF DATA AFTER SIX REPLICATES).

With a lower seeding density, there are initially fewer cells to affect the pH. Therefore, the cumulative change in pH over the course of a passage is smaller.

Using a vent cap allows the carbon dioxide to escape from the medium, and the final carbon dioxide partial pressure is approximately 60 mbar lower than normal. As the carbon dioxide normally reacts with water to form a weak acid, which lowers the pH of the medium, the reduction in carbon dioxide allows the pH of the supernatant to remain more stable.

Cultivation at 35°C compared to 37°C possibly slows the metabolism of the cells with lower glucose and oxygen utilisation for the same number of cells than under normal conditions. Correspondingly, the overall decrease observed in pH is also lower.

5.2 PRODUCTION RESULTS

The aim of the experiments in production phase was to determine the effect of pH and perfusion rate on the glycosylation.

The extent of glycosylation was monitored by measuring the extent of sialylation, which is determined by the charge profile. The charge profile is an expression of the amount of neutral, mono-, di- and tri-sialylated glycans and is characterised by the Z-number. As the Z-number decreases during downstream processing, it is preferred to have an average Z-number of 180 or higher at the start of DSP to ensure that the final Z-number is within specification. This is referred to as satisfactory glycosylation.

For the experiments, eight runs were performed using three bioreactors and the cells from three different seed trains. The pH can be kept constant over a run. However, during a run the perfusion rate needs to be increased to keep the glucose level above 1.0 g/L. The perfusion rate can therefore only be specified at the start of the production phase, and is then generally kept constant for the first ten days until it needs to be increased to increase the glucose concentration. To consider this, the data generated from these experiments was analysed in two ways:

1. By run: analysis of variance (ANOVA) on the initial conditions of each run and the average Z-number per run.
2. By harvest: the analysis of covariance (ANCOVA) on the continuous data from each run.

The results of the ANOVA and ANCOVA will be presented in the following sections.

5.2.1 SOURCES OF VARIATION

Two important sources of variation were identified in the data: the cell counting and the analytical method used to determine the Z-number.

As has already been observed in the seed train, cell counts often vary between process operators. The same phenomenon was observed in the bioreactors with cell counts sometimes varying significantly from day to day, as seen in Figure 18. This formed a significant source of variation, especially for the ANCOVA analysis, which was based on semi-continuous data.

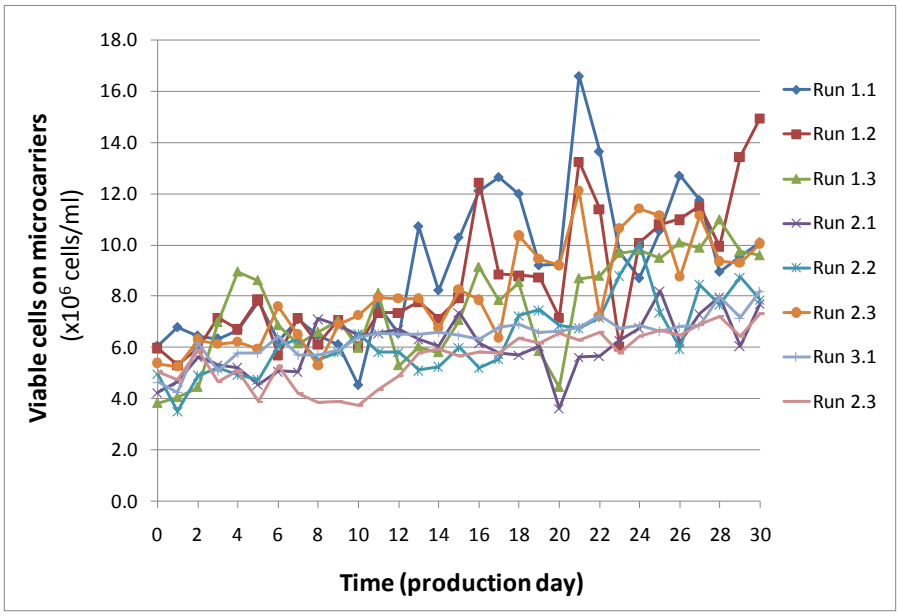

FIGURE 18. CONCENTRATION OF VIABLE CELLS ON MICROCARRIERS FOR EACH RUN DURING PRODUCTION PHASE.

Due to the decrease in Z-number during DSP processing, it was decided to analyse the glycosylation and calculate the Z-number for these experiments before the first chromatography step. This meant that there were more impurities than usual in the harvests. The increase in impurities was reflected by the relatively large standard deviation of the analysis method. The standard deviation of the Z-number, calculated over 8 repeated measurements of the same harvest before the first chromatography step was 9, compared to a standard deviation of 3 over 8 repeated measurements of the same harvests after the first two chromatography steps.

The large deviation meant that almost all the Z-number data points fell within two standard deviation of the average Z-number over each run. Consequently, the Z-number appears almost constant over each run; the analysis method does not appear sensitive enough to detect significant smaller changes during the course of the process. This substantiates the assumption of treating data as an average, but is in contrast with some of the sparse historical data available on the glycosylation over the course of a run where the glycosylation appears to change over the course of a run. Therefore, data will still also be analysed per harvest.

5.2.2 ANOVA PER RUN

One way of approaching the analysis of the data generated from these experiments is to consider the glycosylation as stable over a run and work with the average data; this also allows one to test the effect of initial parameters, like the initial perfusion rate. An ANOVA of the effect of pH and the initial perfusion rate on the average Z-number of each run (as a measure of the extent of glycosylation) was performed using Statistica™ with an alpha value of 0.05.

pH

The ANOVA of pH and initial perfusion rate revealed that the pH does not have a significant effect on the average Z-number. That the pH does not have statistically significant effect on the glycosylation levels of the product disproves the hypothesis that better glycosylation would be seen at pH 6.9 than at pH 6.7. It would thus appear that the glycosylation of this protein in these cells is stable in the pH range specified for this process.

Perfusion rate

In contrast to the pH, the perfusion rate does have an effect on the Z-number. To further investigate the effect of the perfusion rate on the glycosylation, an ANOVA was performed with the initial perfusion rate, size of the increase in perfusion rate during the course of the culture, the rate of increase in perfusion rate over the course of the culture and the method of increase (either linearly, or as required by the culture). These settings can be seen in Table 5.

TABLE 5. ASPECTS OF PERFUSION INVESTIGATED AND RESULTING AVERAGE Z-NUMBER

Run	Initial perfusion rate	Size of increase in perfusion rate	Rate of increase in perfusion rate	Increase type	Average Z-number
Run 1.1	High	Low	High	High	173
Run 1.2	High	High	High	High	177
Run 1.3	Low	High	High	High	182
Run 2.1	Low	High	High	High	180
Run 2.2	Low	High	Low	Low	172
Run 2.3	High	Low	Low	High	173
Run 3.1	Low	High	Low	High	188
Run 3.2	Low	High	Low	High	192

This ANOVA revealed that the initial perfusion rate and the method of increase both appear to have a significant effect on the average Z-number of each run. Interestingly the variation in the average Z-number of runs that had the same perfusion conditions (runs 1.2 & 2.1 and runs 3.1 & 3.2) is much smaller than the standard deviation of the analysis method.

As the Z-number decreases during downstream processing, it is preferred to have an average Z-number of 180 or higher at the start of DSP to ensure that the final Z-number is within specification. This is referred to as satisfactory glycosylation.

Figure 19 shows the average Z-number obtained in each run according to the initial perfusion rate. All the runs that gave satisfactory glycosylation results started at a low perfusion rate. The only exception was run 2.2, where the perfusion rate was increased linearly rather than as required to keep the glucose concentration above 1.0 g/L, as it was for the other runs.

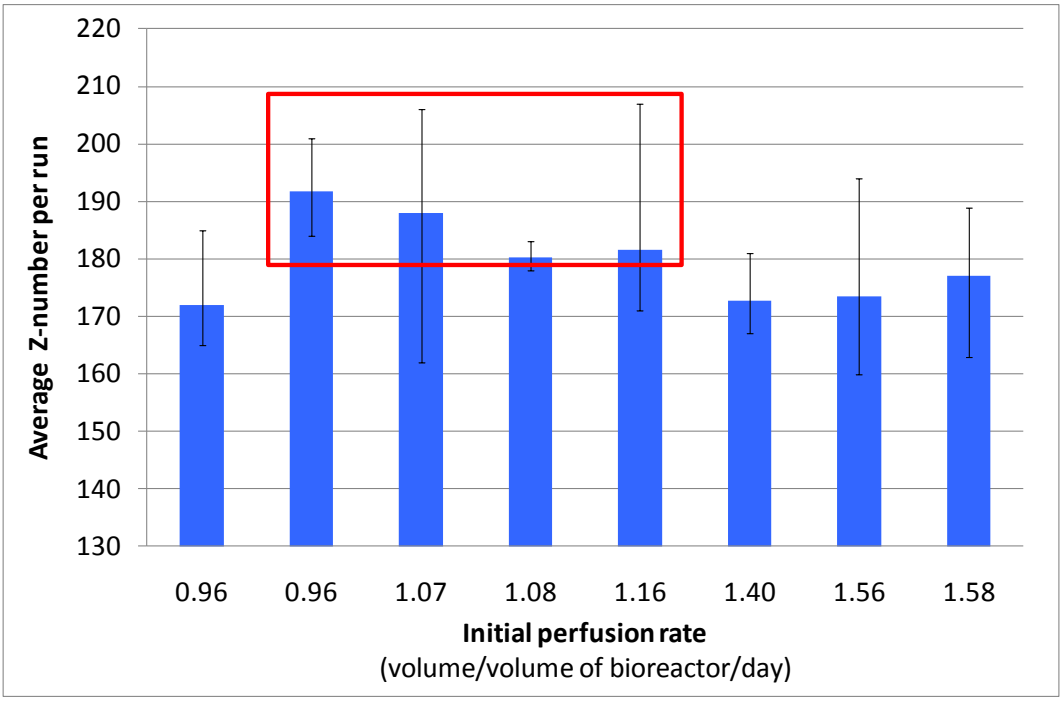

FIGURE 19. AVERAGE Z-NUMBER PER RUN FOR EACH INITIAL PERFUSION RATE (ERROR BARS REPRESENT DATA RANGE OF Z-NUMBER IN EACH RUN), RUNS THAT GAVE SATISFACTORY Z-NUMBERS (>180) ARE HIGHLIGHTED BY THE RED BOX.

The Z-numbers of the runs in these experiments cannot be directly compared (i.e. Z-number to Z-number) to historic data as the method of analysis was changed at a certain point. However, it is known which historic runs produced harvests that lead to final bulks with satisfactory glycosylation levels (i.e. they were in specification) and this can be compared to the data from these experiments. As seen in Figure 20, historic data shows the same trend, with lower initial perfusion rates leading to satisfactory glycosylation. The only exceptions are run RP33-133, which is discussed further in section 5.2.3, and run 2.2 where the perfusion was increased linearly instead of as required by the cell culture.

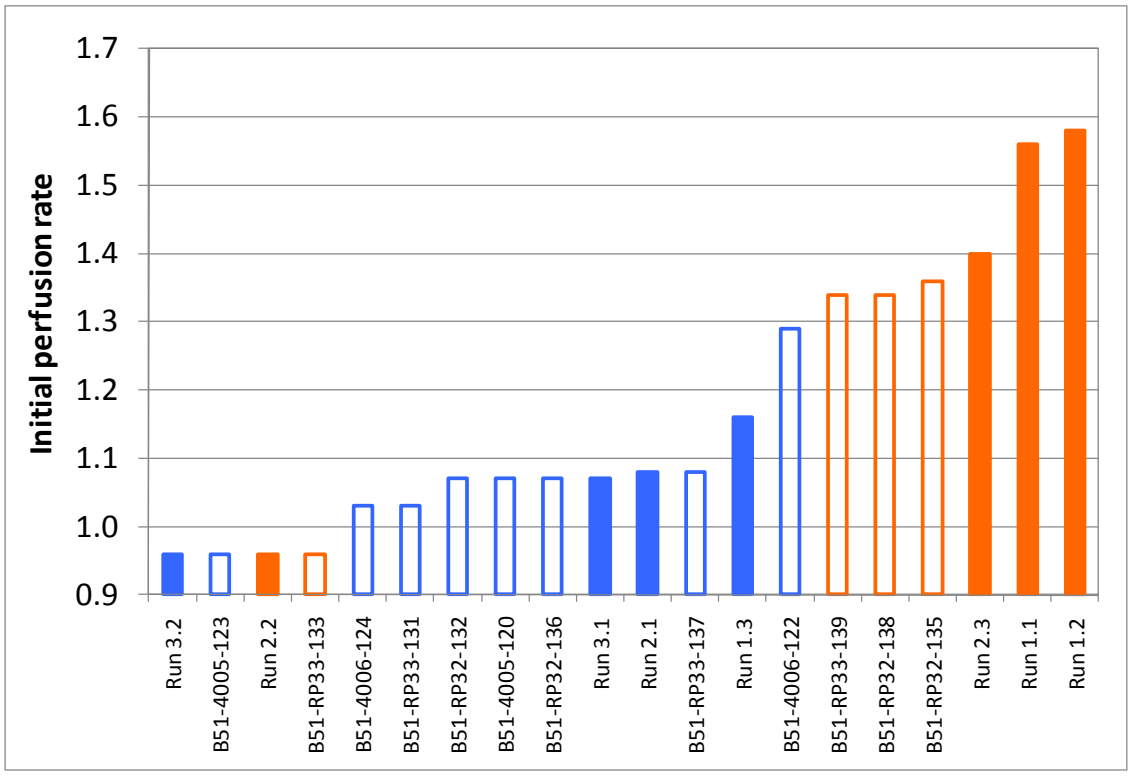

FIGURE 20. INITIAL PERFUSION RATES USED FOR HISTORIC RUNS (EMPTY BARS) AND CURRENT EXPERIMENTAL RUNS (SOLID BARS), BLUE RUNS GAVE SATISFACTORY GLYCOSYLATION AND ORANGE RUNS GAVE UNSATISFACTORY GLYCOSYLATION.

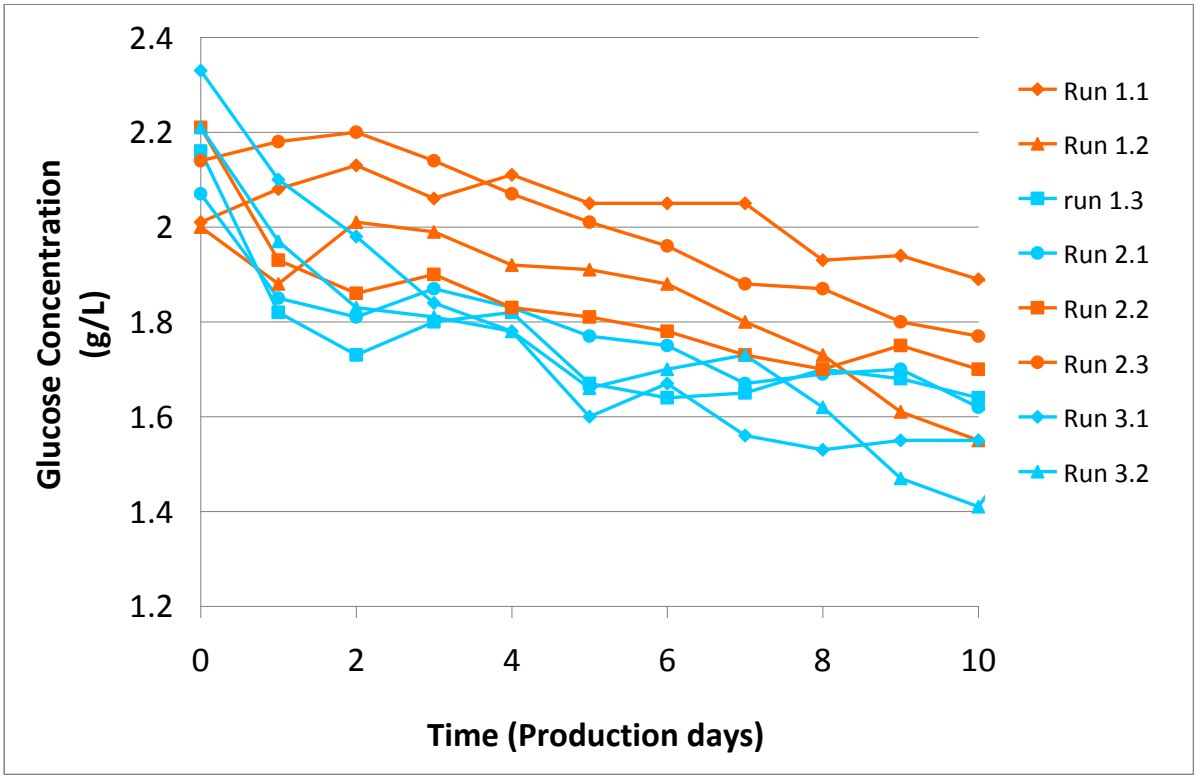

FIGURE 21. GLUCOSE CONCENTRATION DURING THE FIRST TEN DAYS OF PRODUCTION (BLUE CURVES INDICATE RUNS THAT GAVE SATISFACTORY GLYCOSYLATION AND ORANGE CURVES INDICATE RUNS WITH UNSATISFACTORY GLYCOSYLATION).

From Figure 20, it appears the transition from satisfactory to unsatisfactory glycosylation occurs at an initial perfusion rate of between 1.2 – 1.3 v/v/d. Therefore, it is recommended to use an initial perfusion rate of less than 1.2 v/v/d.

The fact that the initial perfusion rate seems to be so important to the glycosylation could either be because of inhibition due to the concentration of certain metabolites, or to the difference in the hydrodynamic forces (i.e. shear stress) on the cells at different perfusion rates. Unlike the perfusion rate and pH, the metabolites do not remain constant during the first ten days, so cannot be analysed based on their average data. The only metabolite where there appears to be a clear difference in the trends of the runs with satisfactory and unsatisfactory glycosylation during the first ten days of production, is the glucose, as can be seen in Figure 21.

Interestingly, the runs with a lower initial glucose concentration gave better glycosylation rather than the runs with a higher perfusion rate. This is in direct contrast with most published data about glycosylation, (Hayter *et al*. 1992 & 1993, Restelli and Butler 2002, Wong *et al*., 2005) where a high glucose concentration is considered essential for good glycosylation. This could be an indication that the better glycosylation at lower perfusion rates is in fact due to the decreased stress on the cells. However, there are no other factors, like an increase in the titre at higher perfusion rates, which would indicate an increase in the processing in the Golgi as a stress response of the cell.

5.2.3 ANCOVA PER HARVEST

Analysis of co-variance (ANCOVA) allows one to perform analysis of variance on continuous data and allows for the incorporation of supplementary data. An ANCOVA was performed on all the data from each harvest, for which the glycosylation had been measured, for correlation to the Z-number (as an indication of the extent of glycosylation), using Statistica™ and a confidence level of 95%. The results of the ANCOVA showed that the specific perfusion rate, the specific glucose consumption rate, the specific lactate concentration and the specific glutamine concentration were all correlated to the Z-number of the harvest.

All these factors have a high degree of variability given the variability in the cell count and the variability in the analytical method used to determine the Z-number. The variability made it difficult to draw correlations for the effect of these parameters on the Z-number. This can be seen in Figure 22, which shows the correlation between the Z-number and the specific perfusion rate. From Figure 22, it would appear that there might be a trend toward better glycosylation, and thus higher Z-numbers, at higher specific perfusion rates but the variability is such that it is not possible to draw any conclusions. It is also not possible to make a statistically significant linear correlation, as indicated by the R^2 value of 0.12.

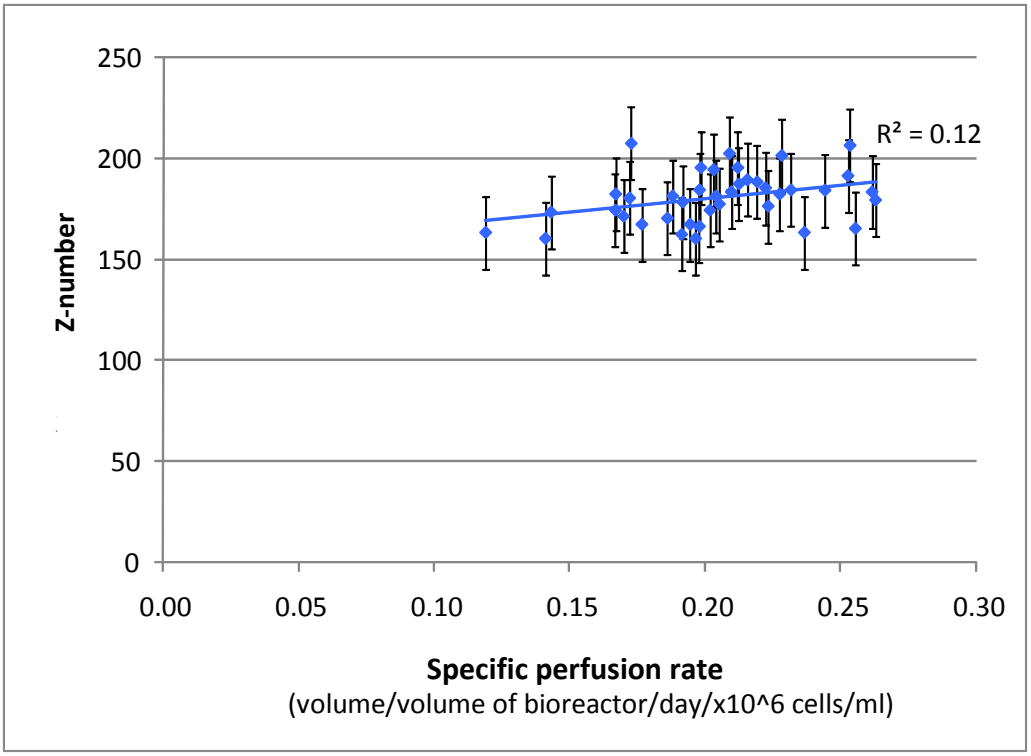

FIGURE 22. Z-NUMBER FOR EACH HARVEST CORRELATED TO SPECIFIC PERFUSION RATE (ERROR BARS ARE TWO STANDARD DEVIATIONS OF THE ERROR OF THE ANALYTICAL METHOD USED TO DETERMINE THE Z-NUMBER).

On the other hand, when compared to historic data, as shown in Figure 23, the specific perfusion rate appears to be a possible reason for the decreased Z-number. Both historic runs RP33-133 (which started at a low initial perfusion rate, see Figure 20) and run RP32-135 had unusually high specific perfusion rates, as seen in Figure 23.

Though the perfusion rates in Figure 22 and Figure 23 represent the same aspect of the process, they were calculated in slightly differently ways. The specific perfusion rate in Figure 22 was calculated as *perfusion/cell concentration* with the specific perfusion rate in Figure 23 was calculated as *perfusion/total cells in bioreactor*, to take into account slight difference between bioreactor volumes from historic data.

The importance of the specific perfusion rate adds weight to the theory that the hydrodynamic forces on the cells, which would increase with increased perfusion rate, have an influence on the glycosylation of the protein probably through a mechanism related to the stress on the cells. However, this is the first time this theory has been proposed so it is not possible to compare these results to those of other authors and defining the mechanism was beyond the scope of this study.

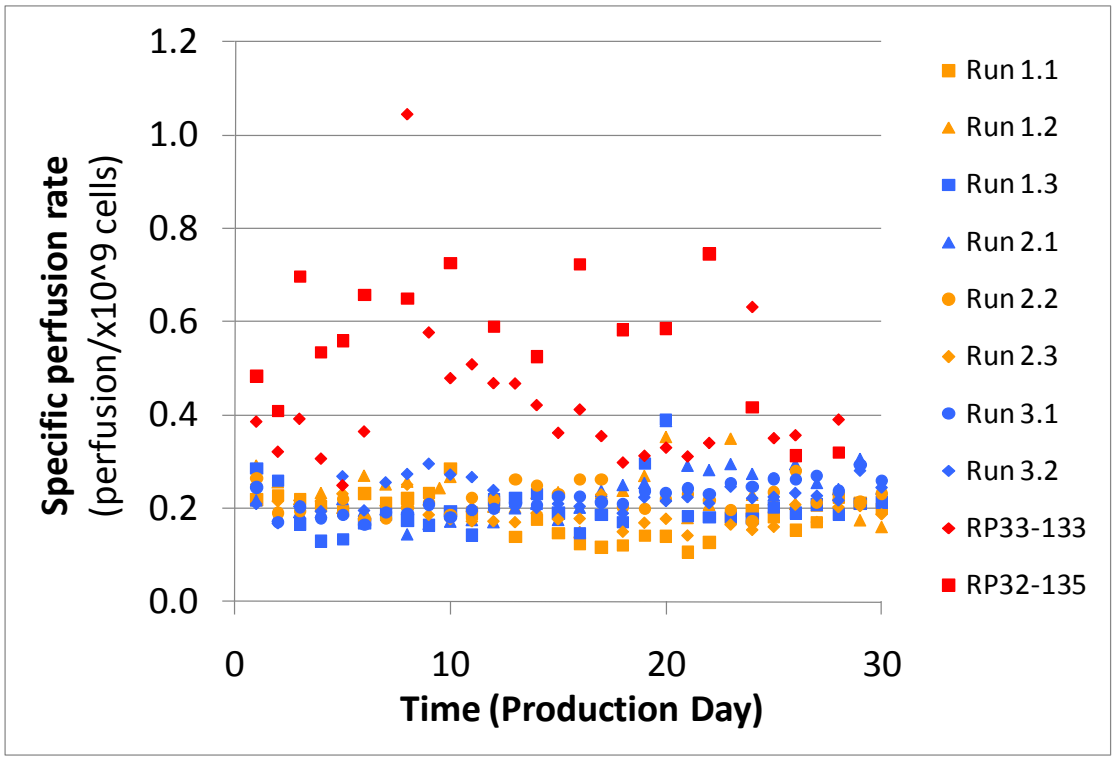

FIGURE 23. SPECIFIC PERFUSION RATE DURING PRODUCTION (RED MARKERS INDICATE HISTORIC RUNS WITH UNSATISFACTORY GLYCOSYLATION, BLUE MARKERS INDICATE EXPERIMENTAL RUNS WITH SATISFACTORY GLYCOSYLATION AND ORANGE MARKERS INDICATE EXPERIMENTAL RUNS WITH UNSATISFACTORY GLYCOSYLATION).

It would also seem that the correlation between the specific perfusion rate and the Z-number of the proteins is non-linear over the entire range of historic and new data. From the new data, it would appear that increasing the specific perfusion rate gives better glycosylation, while from historic data it appears that increasing the specific perfusion rate too much results in unsatisfactory glycosylation. Further research over the whole range of specific perfusion rates displayed in this data would be required to determine the correct correlation. However, in the meantime a limit to the specific perfusion rate of 0.3 medium volume/bioreactor volume/day/10^9 cells could be imposed to limit the negative effects of high specific perfusion rates.

The other factors that were shown to be significant from the ANCOVA (data shown in Figure 24 - Figure 26) do not find similar substantiation in historic data. It was also not possible to establish a statistically significant linear correlation to the Z-number for these factors either, as shown by the very low R^2 values. This could be due to either the variation in both the cell counts and method of analysis, as discussed earlier, or an indication that they are actually non-linear correlations.

The correlation between specific glucose consumption rate and Z-number, shown in Figure 24, might indicate that better glycosylation is achieved at higher glucose consumption rates. This is logical as glucose provides most of the precursors required for building glycans and is the cell's main source of energy. However, it is difficult to compare these trends to literature as most authors only report the glucose concentration in the supernatant not the specific glucose consumption rate (Hayter et al. 1992 & 1993, Wong et al., 2005).

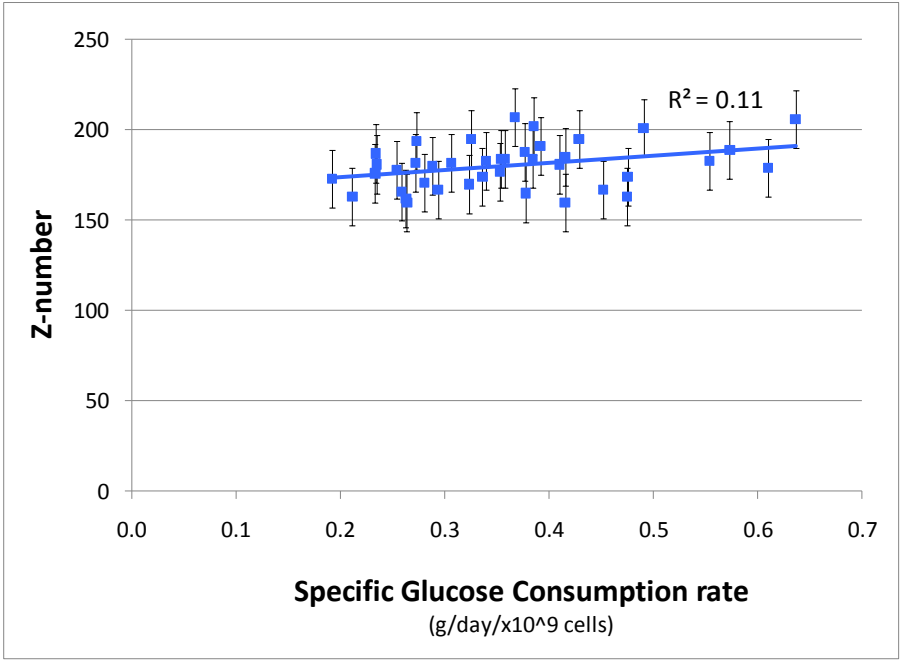

FIGURE 24. Z-NUMBER PER HARVEST CORRELATED TO SPECIFIC GLUCOSE CONSUMPTION RATE (ERROR BARS ARE TWO STANDARD DEVIATIONS OF THE ERROR OF THE ANALYTICAL METHOD USED TO DETERMINE THE Z-NUMBER).

The importance of the glutamine concentration rather than the consumption rate corresponds more to the result expected from literature than the glucose consumption rate. In this case, it is probable that glutamine is important for its value in supplying certain precursors required for glycosylation (Hayter *et al*. 1992 & 1993, Butler and Restelli, 2000, Wong *et al*., 2005) rather than as an energy source for the cells.

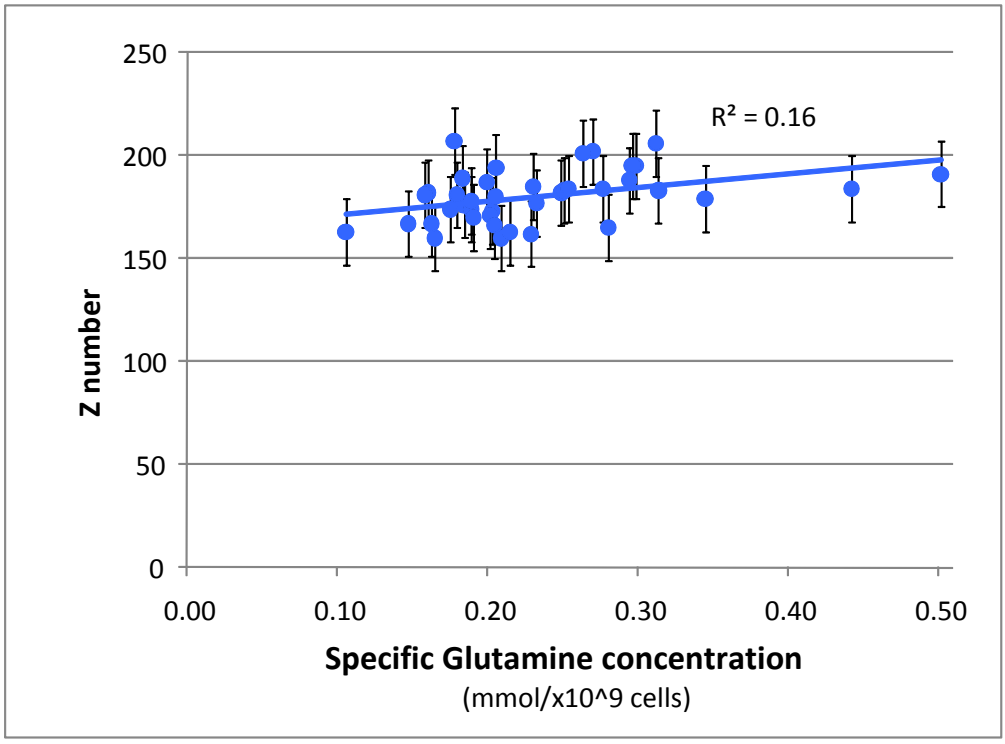

FIGURE 25. Z-NUMBER PER HARVEST CORRELATED TO SPECIFIC GLUTAMINE CONCENTRATION (ERROR BARS ARE TWO STANDARD DEVIATIONS OF THE ERROR OF THE ANALYTICAL METHOD USED TO DETERMINE THE Z-NUMBER).

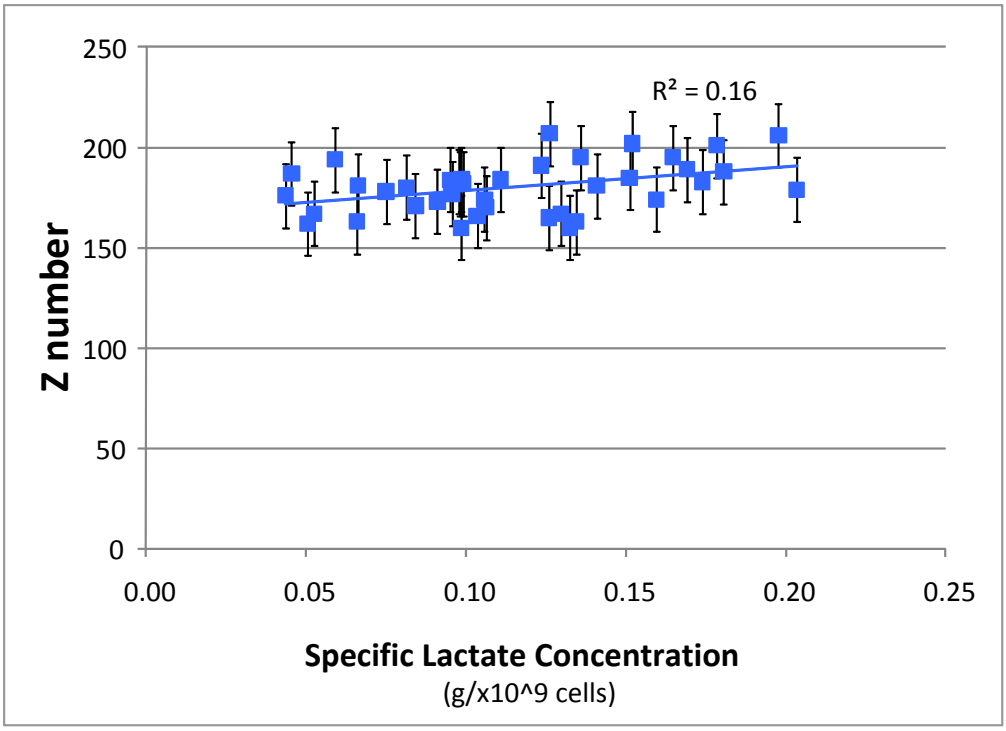

FIGURE 26. Z-NUMBER PER HARVEST CORRELATED TO SPECIFIC LACTATE CONCENTRATION (ERROR BARS ARE TWO STANDARD DEVIATIONS OF THE ERROR OF THE ANALYTICAL METHOD USED TO DETERMINE THE Z-NUMBER).

The correlation between the specific lactate concentration and the Z-number can be seen in Figure 26. No literature could be found to suggest that lactate has any effect on the extent of glycosylation. The specific lactate concentration was probably found to be significantly correlated to the Z-number because it also correlates to the specific glucose consumption rate, as glucose is always metabolized to lactate in a specific molar ratio.

None of the factors indicated by the ANCOVA to be significantly correlated to the Z-number show very good linear correlations. Part of this is probably due to the variation in the cell counting and the variation in the glycan-analyses, but it could also be that the correlations are not linear as has been shown for the specific perfusion rate and ANCOVA assumes a linear relationship between components. All the factors shown to be significantly correlated to the Z-number are also significantly correlated to each other and the glycosylation is probably affected by a combination of these factors.

It is possible that the relationships between factors are linear within certain ranges and with further research, it is entirely possible that models could be developed to predict the level of glycosylation occurring in the process based on these factors. This research conducted in this study, then represents the first step in understanding the factors that influence the glycosylation in this process and in using them to predict the outcome of the glycosylation.

6 CONCLUSIONS AND RECOMMENDATIONS

From the above results and discussion, the following conclusions and recommendations can be made for the seed train and the production phase.

6.1 SEED TRAIN

The aim of these experiments on the seed train was to determine what the influence of the controlled process parameters was on the cell growth and final PDL and whether any of these was the cause of the apparent increase in final PDL over the past several campaigns. The medium volume, seeding density, cultivation temperature, aeration and medium feed temperature were all chosen for investigation based on a literature survey and process considerations. The following hypotheses were made:

- Decreasing the medium volume will yield a lower PDL due to slower cell growth caused by lower glucose availability.
- Decreasing the seeding density will yield a higher PDL.
- Decreasing the temperature will decrease the growth rate and yield a lower PDL.
- There will be no significant difference to the cell growth when cold medium is used rather than pre-heated medium.
- Using vent caps will increase the oxygen content of the medium in the roller bottles and the cell growth, yielding a higher PDL.

6.1.1 THE SEEDING DENSITY IS THE ONLY PARAMETER TO INFLUENCE THE FINAL PDL

The seeding density proved to be the only variable to have a significant influence on the final PDL of the cells. The growth of the cells used in this process appears to be limited, once the cells reach confluence at day 5, and thus decreasing the seeding density increases the final PDL, as hypothesised in section 3. Based on this observation it is recommended that a higher seeding density be used to ensure a lower final PDL: 170 - 200 x 10^6 cells per roller bottle in the 1750 cm^2 roller bottles.

However, the seeding density does not appear to be the cause of the apparent increase in the final PDL over the past several campaigns, as historic data shows that all campaigns were inoculated with a high seeding density. As most of the uncontrolled variables, like the source of the growth medium, have been eliminated as the cause, it seems possible that the apparent increase in the final PDL is due to the variation in cell counting between operators as operators were changed between campaigns. It is recommended that a mechanical cell counter be used to verify operators results and as a standard between campaigns.

6.1.2 The 1°C temperature difference found in the incubation chambers does not significantly affect the cell culture

It was hypothesised that decreasing the temperature of the incubation chamber would result in a reduced cell growth rate, and probably a lower PDL. This hypothesis was disproved for the temperature ranges in question.

In this case, a difference was observed in the cells over the 2°C difference of the permitted temperature range (35-37°C): with the cells exhibiting a slight apparent decrease metabolism at 35°C compared to 37°C, but the growth rate and final PDL were not significantly different.

As to the cells cultivated at 36°C and 35°C, no significant difference was observed in the cell growth, the final PDL or the metabolism. It can be concluded that the 0.9°C temperature difference sometimes observed between different areas of the incubation chamber does not significantly affect the cells.

6.1.3 Decreasing the medium volume leads to fewer cells in suspension

It was hypothesised that decreasing the medium volume in the roller bottles would lead to a lower PDL due to decreased availability of nutrients. This was not at all the case, and the most significant results of decreasing the medium volume were far fewer cells in suspension and the appearance of a slightly slower metabolism in the cells.

The decrease in the number of cells in suspension at a lower medium volume is probably due to the change in the size of the shear force of the medium on the cells, which is smaller at a smaller volume compared to a larger volume at the same rotational speed. The decrease in glucose consumption and lactate production is probably because the contact time of the cells with the medium is less at a smaller volume than at a larger volume at the same rotational speed.

6.1.4 THE MEDIUM FEED TEMPERATURE DOES NOT SIGNIFICANTLY INFLUENCE THE CELL CULTURE

It was hypothesised that the temperature of the fresh medium used to inoculate the roller bottles and for medium changes would not significantly influence the cell culture. This was proved to be true in all observed response variables; none of the observed variables were significantly affected by the medium feed temperature. The only exception was the initial oxygen concentration, but as the medium warmed to the desired temperature within a couple of hours of being in the incubator, that too evened out within a couple of hours, and did not significantly affect the cell growth, metabolism or final PDL.

6.1.5 SUMMARY OF SEED TRAIN RESULTS AND RECOMMENDATION

The following table summarises the results from the seed train experiments:

TABLE 6. SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS FROM SEED TRAIN EXPERIMENTS

Hypothesis	Conclusion	Recommendation
Decreasing the medium volume will yield a lower PDL	False	N/A
Decreasing the seeding density will yield a higher PDL	True	Inoculate at a higher seeding density to ensure the PDL remains within specification, use a mechanical cell counter to verify cells counting between operators and campaigns
Decreasing the temperature will yield a lower PDL	False	N/A
No significant difference to the cell growth when cold medium is used at inoculation	True	Further studies into the effect of the cold medium on cell culture parameters not investigated in this study, e.g. enzymes before the decision is taken to change the process
Vent caps will increase the oxygen content of the medium in the roller bottles and yield a higher PDL	Oxygen: true for 850 cm^2 roller bottles PDL: false	N/A

6.2 PRODUCTION IN BIOREACTORS

The aim of the experiments performed in bioreactors during the production phase was to determine the effect of the pH and the perfusion rate on the extent of glycosylation of the product and to determine whether either the pH or the perfusion rate could be the cause of the out-of-specification glycosylation seen in certain final bulks. Following a literature survey, the subsequent hypotheses were made:

- Better glycosylation will be seen at pH 6.9, than at pH 6.7
- A higher perfusion rate will lead to better glycosylation due to increased glucose and glutamine concentrations

6.2.1 pH DOES NOT AFFECT THE GLYCOSYLATION

It was hypothesised that better glycosylation would be seen at pH 6.9 than at pH 6.7. In this process no significant difference was observed between the level of glycosylation at pH 6.9, pH 6.8 and at pH 6.7. The pH range in question is very narrow and though it lies across the end of range recommended by literature, variation within this range does not appear to be the cause of the unsatisfactory glycosylation seen in certain final bulks.

6.2.2 A LOW INITIAL PERFUSION RATE LEADS TO SATISFACTORY GLYCOSYLATION

Based on the above results attained in bioreactors it can be shown that using a lower initial perfusion rate leads to better glycosylation. As a lower perfusion rate means that the glucose and glutamine concentrations considered essential for satisfactory glycosylation are also low it is suggested that the improvement in glycosylation is possibly due to the a decrease in the hydrodynamic forces on the cells rather than the nutrient concentrations.

It is recommended that a lower initial perfusion rate be used for future runs. Based on the data obtained from these experiments and historic data an initial perfusion rate of 1.0 v/v/d is recommended.

6.2.3 ANCOVA

No hypotheses were created for the results of the ANCOVA analysis, as the ANCOVA represented a way of exploiting all the data generated during these experiments on the effect of pH and perfusion rate to gain further understanding of the factors that are important for the glycosylation of the product. The results of the ANCOVA showed that the specific perfusion rate, the specific glucose consumption rate, the specific lactate concentration and the glutamine concentration were all correlated to the Z-number of each harvest.

None of the factors indicated by the ANCOVA to be significantly correlated to the Z-number shows very good linear correlations (ANCOVA assumes a linear relationship between components). Part of this is probably due to the variation in the cell counting and the variation in the glycan-analyses but it could also be that the correlations are not linear.

This has been illustrated for the specific perfusion rate. From the data generated for these experiments it appears that a high specific perfusion rate gives better glycosylation but from historic data is appears that very high specific perfusion rates negatively affect the glycosylation, indicating a non-linear correlation.

The specific perfusion rate was shown to be significant from historic data and is the probable cause of the unsatisfactory glycosylation seen in runs RP33-133 and RP32-135. Further research over the whole range of specific perfusion rates displayed in this data is recommended to determine the correct non-linear correlation; however, in the interim a limit to the specific perfusion rate of 0.3 volumes/day/10^9 cells is proposed to limit the negative effects of high specific perfusion rates.

As to the other factors, the correlations indicate that better glycosylation is achieved at higher specific glucose and glutamine consumption rates and at higher specific lactate concentrations. The importance of glucose and glutamine is probably due to their use as substrates for glycosylation and the importance of the lactate concentration is likely because the glucose is metabolized to lactate is a specific molar ratio.

With further research, it is entirely possible that models could be developed to predict the level of glycosylation occurring in the process based on these factors. The research described here then represents the first step in understanding the factors that influence the glycosylation in the process and in using them to predict the outcome of the glycosylation.

6.2.4 Reasons for the out-of-specification final bulks

Given the above results and discussion, the following reasons are proposed for the unsatisfactory glycosylation seen in the final bulks of certain runs:

- RP33-133 : Very high specific perfusion rate
- RP32-135 : High initial perfusion rate and very high specific perfusion rate
- RP32-138 : High initial perfusion rate
- RP33-139 : High initial perfusion rate

This is a most satisfactory result as previous internal studies of these deviations had not been able to find reasons for these final bulks being out of specification.

7 REFERENCE LIST

105923, 2006: r-hLH serum process Investigation at SBC – Support to LSA

Andersen DC and Goochee CF. 1994. The effect of cell-culture conditions on the oligosaccharide structures of secreted glycoproteins. *Current Opinion in Biotechnology*, 5: 546–549

Andersen DC and Goochee CF. 1995. The effect of ammonia on the O-linked glycosylation of granulocyte colony-stimulating factor produced by chinese hamster ovary cells. *Biotechnology and Bioengineering*, 47(1):96-109

Balin, A.K., Fisher, A.J. and Carter, J.M. 1984. Oxygen modulates growth of human cells at physiologic partial pressures. *The journal of experimental medicine*, 160 (1): 152-166

Bollati-Fogolin M., Forno, G., Nimtz, M., Conrado, H. S., Etcheverrigaray, M. and Kratjie, R. 2005. Temperature reduction in cultures of hGM-CSF-expressing CHO cells: effect on productivity and product quality. *Biotechnology Progress*, 21(1):17-21.

Borys M.C., Linzer, D.I.H and Papoutsakis E. T. 1993. Culture pH affects expression rates and glycosylation of recombinant mouse placental lactogen proteins by Chinese hamster ovary (CHO) cells. *Bio/Technology*, 11:720-724.

Butler M. 2005. Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. *Applied Microbiology and Biotechnology*, 68:283-291.

Chen, P. and Harcum S.W. 2006. Effects of elevated ammonium on glycosylation gene expression in CHO cells. *Metabolic Engineering*, 8(2):123-132

Chotigeat, W., Watanapokasin, S., Mahler S.S. and Gray, P.P. 1994. Role of environmental conditions on the expression levels, glycoform pattern and levels of sialytransferasefor hFSH produced by recombinant CHO cells. *Cytotechnology*, 15:217-221.

Chu L and Robinson D.K. 2001. Industrial choices for protein production by large-scale cell culture. *Current Opinion in Biotechnology*, 12:180–187

Corning Roller bottles – Selection and Use Guise. (2005). Document reference: POD 6/05 CLS-BP-003W-REV1

Curling, E.M.A., Hayter, P.M., Baines, A.J., Bull, A.T., Gull, K., Strange, P.G. and Jenkins, N. 1990. recombinant human interferon-γ: differences in glycosylation and proteolytic processing lead to heterogeneity in batch culture. *Biochemistry Journal*, 242:333-337.

Czermark, P., Portner, R. and Brix A. 2009. Special engineering aspects. *In* Eibl R., Eibl D., Portner R., Catapano G. and Czermark, P. (eds). *Cell and Tissue Reaction Engineering*. Berline-Heidelberg: Springer-Verlag. p.83-172

Fernandes, D. 2006. Biopahrmaceutical Sialylation. *European Biopharmaceutical review*, 100-104, Spring.

Gawlitzeck, M., Valley, U., Nimtz, M., Wagner R. and Conradt, H.S. 1995. Characterization of changes in the glycosylation pattern of recombinant proteins from BHK-21 cells due to different culture conditions. *Journal of Biotechnology*, 42:117-131.

Gramer, M. J. 1999. Measurement of cell-culture glycosidase activity. *In* Jenkins, N. (ed), *Animal cell biotechnology: methods and protocols.* Totowa NJ: Humana Press. p289-298.

Gstraunthaler G, Seppi T and Pfaller W. 1999. Impact of Culture Conditions, Culture Media Volumes, and Glucose Content on Metabolic Properties of Renal Epithelial Cell Cultures. *Cellular physiology and biochemistry* 9(3):150-72.

Hayter P. M., Curling E. M. A., Gould, M. L., Baines A. J., Jenkins, N., Salmon, I., Strange, P. G. and Bull, A. T. 1991. Chinese hamster ovary cell growth and interferon production kinetics in stirred batch culture. *Applied Microbiology and Biotechnology*, 34(5):559-564.

Hayter P. M., Curling E. M. A., Gould, M. L., Baines A. J., Jenkins, N., Salmon, I., Strange, P. G. and Bull, A. T. 1992. Glucose-limited chemostat culture of Chinese hamster ovary cells producing recombinant human intereferon-γ. *Biotechnology and Bioengineering*, 39:327-335.

Hayter P. M., Curling E. M. A., Gould, M. L., Baines A. J., Jenkins, N., Salmon, I., Strange, P. G. and Bull, A. T. 1993. The effect of dilution rate on CHO cell physiology and recombinant interferon-γ production in glucose-limited chemostat culture. *Biotechnology and Bioengineering*, 42:1077-1085.

Hughes B. and Hann L.E. 2007. The Production of Biopharmaceuticals. *In* Boehncke, W.-H.; Radeke, H.H. (Eds.) *Biologics in general medicine*. Springer: p60-66.

Hunt L, Hacker DL, Grosjean F, De Jesus M, Uebersax L, Jordan M and Wurm FM. 2005. Low-temperature pausing of cultivated mammalian cells. *Biotechnology and Bioengineering* 89(2):157-163

Jayapal KP, Wlaschin KF and Hu W. 2007. Recombinant Protein Therapeutics from CHO Cells - 20 Years and Counting. *Chemical Engineering Progress*, 103(10):40-47

Jenkins N., Parekh, R. B. and James D. C. 1996. Getting the glycosylation right: implications for the biotechnology industry. *Nature biotechnology*, 14: 975-981.

Khmelnitsky YL. 2004. Current strategies for in vitro protein glycosylation. *Journal of Molecular Catalysis B: Enzymatic* 31:73–81

Kimura R. and Miller W. M. 1997. Glycosylation of CHO-derived recombinant tPA produced under elevated pCO2. *Biotechnology Progress*, 13(3):311-317.

LU-P-000009, 2005: Glycan Mapping Deviation for r-hLH Intermediate Report

LU-P-000010, 2006: Glycan Mapping Deviation for r-hLH Final Report

Lu S, Sun X and Zhang Y. 2005. Insight into metabolism of CHO cells at low glucose concentration on the basis of the determination of intracellular metabolites. *Process biotechnology,* 40(5):1917-1921

Lyn AA and William MW. 1992. CHO cell responses to low oxygen: Regulation of oxygen consumption and sensitization to oxidative stress. *Biotechnology and Bioengineering,* 40(4):505-516

Nyberg GB, Balcarcel RR, Follstad BD, Stephanopoulos and Wand IC. 1999. Metabolic Effects on Recombinant Interferon-g Glycosylation in Continuous Culture of Chinese Hamster Ovary Cells. *Biotechnology and Bioengineering*, 62(3):336-347

Papoutsakis ET. 1991. Fluid-mechanical damage of animal cells in bioreactors. *Tibtech* 9: 427–437

Perry, R.H.; Green, D.W. (Eds.) 1997. *Perry's Chemical Engineers' Handbook* (7th Edition). McGraw-Hill.

Rasmussen JR. 1992. Effect of glycosylation on protein function. *Current opinion in structural biology.*

Restelli V and Butler M. 2002. The effect of cell culture parameters on protein glycosylation. *In* Al-Rubeai M. (ed.). *Cell Engineering.* Netherlands: Kluwer Academic Publishers, p. 61-92

Restelli, V., Wang, M., Huzel, N., Ethier, M., Perreault, H. and Butler M. 2006. The effect of dissolved oxygen on the production and glycosylation profile of recombinant human erythropotein produced from CHO cells. *Biotechnology and bioengineering*. 94(3):481-494

Rodriguez EN, Perez M, Ordaz Y, Casanova PR, Martinez L and Herrera N. 2005. Experimental factorial design for the study of some variables in roller bottles cell culture. *In* Godia F and Fussenegger M (eds.), *Animal Cell Technology meets Genomics*. Netherlands: Springer, p. 641-643.

Rössler B, Lübben H and Kretzmer G. 1996. Temperature: a single parameter for process optimization in fed-batch cultures of recombinant Chinese hamster ovary cells. *Enzyme and Microbial Technology*. 18:423-427

Rothman RJ, Warren L, Vliegenthart JFG and Hard KJ. 1989. Clonal Analysis of the Glycosylation of Immunoglobulin G Secreted by Murine Hybridomas. *Biochemistry*, 28:1377-1384

Ryan JM, Sharf BB and Cristofalo VJ. 1975. The influence of culture media volume on cell density and lifespan of human diploid fibroblasts. *Experimental Cell Research*. 91(2):389-392

Senger R. S. and Karim M. N. 2003. Effect of shear stress on intrinsic CHO culutre state and glycosylation of recombinant tissue-type plaminogen activator protein. *Biotechnology Progress*, 19:1199-1209.

Sonna LA, Fujita J, Gaffin LA and Lilly GM. 2002. Invited Review: Effects of heat and cold stress on mammalian gene expression. *Journal of Applied Physiology*. 92:1725-1742

Strober W. 2001. Trypan blue exclusion test of cell viability. *Current protocols in immunology*, May;Appendix 3:Appendix 3B

Taylor W.G., Camalier R.F and Stanford K.K. 1978. Density-dependent effects of oxygen on the growth of mammalian fibroblasts in culture. *Journal of Cellular Physiology*, 95(1):33-40

Trummer, E., Fauland, K., Seidunger, S., Schriebl, K., Lattenmayer, C., Kunert, R., Vorauer-Uhl, K., Weik, R., Borth, N., Katinger, H. and Müller, D. 2006b. Process parameter shifting: Part II. Biphasic Cultivation – a toll for enhancing the volumetric productivity of batch processes using EPO-Fc expressing CHO cells. *Biotechnology and Bioengineering*, 94(6):1045-1052.

Trummer, E., Fauland, K., Seidunger, S., Schriebl, K., Lattenmayer, C., Kunert, R., Vorauer-Uhl, K., Weik, R., Borth, N., Katinger, H. and Müller, D. 2006a. Process parameter shifting: Part I. Effect of DOT, pH, and temperature on the performance of Epo-Fc expressing CHO cells cultivated in controlled batch bioreactors. *Biotechnology and Bioengineering*, 94(6):1033-1044.

Tsao EI, Bohn MA, Omstead DR and Munster MJ. 1992. Optimization of a Roller Bottle Process for the Production of Recombinant Erythropoietin. *Annals of the New York Academy of Sciences. Biochemical Engineering VII: Cellular and Reactor Engineering*. 665:127 – 136

Unger D. R., Muzzio, F. J., Aunins J. G., Singhvi, R. 2000. Computational and experimental investigation of flow and fluid mixing in the roller bottle bioreactor. *Biotechnology and Bioengineering*, 70(2):117-130

Werner, R. G., Noé, W., Kopp, K. and Schülter, M. 1998. Appropriate mammalian expression systems for biopharmaceuticals. Drug research, 48:870-880.

Werner RG, Kopp K, Schlueter M. 2007. Glycosylation of therapeutic proteins in different production systems. *Acta Paediatrica Supplement*, 96(455):17-22

Wong D. C. F., Wong, K. T. K., Goh L. T., Heng C. K. and Yap M. G. S. 2005. Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures. *Biotechnology and Bioengineering*, 89(2):164-177

Woo, S. A., Jeon, J., Jeong, Y., Lee S. J. and Yoo, S. K. 2008. Effect of culture temperature on erythropoietin production and glycosylation in a perfusion culture of recombinant CHO cells. Biotechnology and Bioengineering, 101(6):1234-1244

Wu F, Reddy K, Nadeau I, Gilly J, Terpengeningz S, Clanton DJ. 2005. Optimization of a MRC-5 cell culture process for the production of a smallpox vaccine. *Cytotechnology*. 49:95-107

Wurm FM. 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nature Biotechnology*, 22(11):1393-1398

Yoon, S. K., Song, J. Y. and Lee, G. M. 2003. Effect of low culture temperature on specific productivity, transcription level and heterogeneity of erythropoietin in Chinese hamster ovary cells. *Biotechnology and Bioengineering*, 82(3):289-198.

8 APPENDIX

8.1 SAMPLE CALCULATIONS

8.1.1 CALCULATION OF POPULATION DOUBLING LEVEL (PDL)

The population doubling level (PDL) is the number of times the population of cells has doubled while in culture. PDL uses the assumption that each cell producing two daughter cells. Thus at a certain PDL, each of the viable cells present at inoculation, will have produced 2^{PDL} cells.

The total number of cells at a certain time point is then:

$$Final\ total\ cells = initial\ viable\ cells \times 2^{PDL}$$

Therefore:

$$2^{PDL} = (final\ total\ cells / initial\ viable\ cells)$$
$$PDL \times log\ (2) = log\ (final\ total\ cells / initial\ viable\ cells)$$
$$PDL = log\ (final\ total\ cells / initial\ viable\ cells) / log\ (2)$$
$$\mathbf{PDL = log\ (final\ total\ cells / initial\ viable\ cells) \times 3.32}$$

8.1.2 CALCULATION OF PREDICTED FINAL PDL

This section details the calculations of the predicted final PDL used for the discussions in section Table 7.

As explained in section 4.2, the seed train consists of five passages. The final PDL is the cumulative PDL over these five passages, so each passage contributes to the final PDL. For each passage, the number of viable cells initially used to inoculate the passage as well as the total number of cells harvested at the end of the passage influence the PDL (as explained in 8.1.1). However, only the number of viable cells used to inoculate the passage (the seeding density) can be directly controlled by the operators.

The seeding density of the first two passages is pre-determined. The inoculation of the first passage is fixed as all the cells from an ampoule from the cell bank. The PDL of the cells in the ampoule from the cell bank is always 61.

The inoculation of the second passage is fixed as all the cells from the T-flasks at the end of the first passage. The range of the cumulative PDL at the end of the first two passages can be determined from historic data (averaged from the data of 40 seed trains) as 67 ± 1.

That leaves the last three passages in the 1750 cm^2 roller bottles where the seeding density can be adjusted. The seeding density of the 1750 cm^2 roller bottles is 150-200 x 10^6 cells and the range of the number of cells harvested from the 1750 cm^2 roller bottles (determined from the average data from 40 seed trains) is 1650 ± 150 x 10^6 cells.

From this data, it is possible to make a theoretical prediction of the final PDL at the end of the seed train. The PDL for one passage, with varying inoculation and final cell counts, was calculated using the data mentioned in the previous paragraphs and the equation from section 8.1.1. The results can be seen in Table 7. The calculated PDL at a high yield was added to the high historic PDL (i.e. 66) to give the overall high scenario and used for the upper limits of the error bars on the curve in Figure 8. This covered the extremes and all other combinations of high and low yields would most probably fall within the range covered by the error bars.

TABLE 7. RESULTS OF PDL CALCULATIONS FOR ONE PASSAGE IN A 1750 CM^2 ROLLER BOTTLE AT VARYING INOCULATION AND FINAL CELL COUNTS

Inoculation (x 10^6 cells)	PDL at low cell yield at harvest (1500 x 10^6 cells)	PDL at medium cell yield at harvest (1650 x 10^6 cells)	PDL at high cell yield at harvest (1800 x 10^6 cells)
150	3.3	3.5	3.6
160	3.2	3.4	3.5
170	3.1	3.3	3.4
180	3.1	3.2	3.3
190	3.0	3.1	3.2
200	2.9	3.0	3.2

These results were then multiplied by three (for each of the three passages in 1750 cm^2 roller bottles) and added to the historic PDL obtained after the first two passages to reach the predicted final PDL.

The calculated PDL at a low cell yield at harvest was added to the low historic PDL (i.e. 66) to give the overall low scenario and used for the lower limits of the error bars on the curve in Figure 8. The calculated PDL at a medium cell yield at harvest was added to the medium historic PDL (i.e. 67) to give the overall medium scenario and used for the curve in Figure 8. The calculated PDL at a high cell yield at harvest was added to the high historic PDL (i.e. 68) to give the overall high scenario and used for the upper limits of the error bars on the curve in Figure 8. This covered the extremes and all other combinations of high and low yields would fall within the range covered by the error bars.

8.1.3 Z-NUMBER CALCULATIONS

The glycosylation was characterised by determining the glycan charge profile of each harvest – i.e. the amount of neutral, mono-sialylated, di-sialylated, and tri-sialylated glycan species. Glycans from concentrated harvests were enzymatically detached from proteins using PNGase, then labelled with 2-aminobenzamide before HPLC analysis to determine their respective charges (Fernandes, 2006). The percentage of each species was then used to calculate the Z-number for each harvest as follows:

Z = 300 – 3 x %neutral species – 2 x %mono-sialylated species - %di-sialylated species

The rationale behind the equation is confidential as it relates to the particular protein being produced.

8.2 ANOVA FOR EXPERIMENTS IN 850 CM^2 ROLLER BOTTLES

TABLE 8. ANOVA FOR TOTAL CELLS HARVESTED AT THE END OF EACH PASSAGE IN 850 CM^2 ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:Total adherent cells harvested; R-sqr=.56386; Adj:.38214 (Design: 2**(5-0) design (Spreadsheet13) in Work
5 factors at two levels; MS Residual=53231E11
DV: Total adherent cells harvested

Factor	SS	df	MS	F	p
(1)Aeration	1.252774E+1	1	1.252774E+1	2.35348	0.15093
(2)Cultivation Volume	5.289867E+1	1	5.289867E+1	0.99376	0.33850
(3)Inoculum concentration	4.285840E+1	1	4.285840E+1	0.08051	0.78143
(4)Feed temperature	2.004585E+1	1	2.004585E+1	0.37658	0.55089
(5)Cultivation Temperature	2.227247E+1	1	2.227247E+1	4.18414	0.06337
Error	6.387671E+1	12	5.323059E+1		
Total SS	1.464594E+1	17			

TABLE 9. ANOVA FOR VIABILITY OF THE CELLS AT THE END OF EACH PASSAGE IN 850 CM^2 ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:Final viability; R-sqr=.0958; Adj:0. (Design: 2**(5-0) design (Spreadsheet13) in Workbo
5 factors at two levels; MS Residual=.0002002
DV: Final viability

Factor	SS	df	MS	F	p
(1)Aeration	0.00008	1	0.00008	0.40299	0.53746
(2)Cultivation Volume	0.00000	1	0.00000	0.00333	0.95492
(3)Inoculum concentration	0.00002	1	0.00002	0.11990	0.73513
(4)Feed temperature	0.00000	1	0.00000	0.00749	0.93244
(5)Cultivation Temperature	0.00003	1	0.00003	0.16319	0.69333
Error	0.00240	12	0.00020		
Total SS	0.00265	17			

TABLE 10. ANOVA FOR POPULATION DOUBLING LEVEL AT THE END OF EACH PASSAGE IN 850 CM^2 ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:NPD; R-sqr=.84421; Adj:.77929 (Design: 2**(5-0) design (Spreadsheet13) in Workbo
5 factors at two levels; MS Residual=.128889
DV: NPD

Factor	SS	df	MS	F	p
(1)Aeration	0.00747	1	0.00747	0.0580	0.81371
(2)Cultivation Volume	0.08221	1	0.08221	0.6379	0.43999
(3)Inoculum concentration	5.95002	1	5.95002	46.1639	0.00001
(4)Feed temperature	0.48509	1	0.48509	3.7636	0.07624
(5)Cultivation Temperature	0.09572	1	0.09572	0.7426	0.40570
Error	1.54666	12	0.12888		
Total SS	9.92779	17			

TABLE 11. ANOVA FOR THE CHANGE IN THE PH OF THE MEDIUM DURING THE COURSE OF A PASSAGE IN 850 CM^2 ROLLER BOTTLES, ALPHA = 0.05

	ANOVA; Var.:pH change; R-sqr=.80151; Adj:.7188 (Design: 2**(5-0) design (Spreadsheet13) in Workbo 5 factors at two levels; MS Residual=.0303973 DV: pH change				
Factor	SS	df	MS	F	p
(1)Aeration	0.27308	1	0.27308	8.9839	0.01112
(2)Cultivation Volume	0.00530	1	0.00530	0.1744	0.68354
(3)Inoculum concentration	0.37920	1	0.37920	12.4748	0.00413
(4)Feed temperature	0.08268	1	0.08268	2.7201(	0.12500
(5)Cultivation Temperature	0.39958	1	0.39958	13.1453(	0.00347
Error	0.36476	12	0.03039		
Total SS	1.83767	17			

TABLE 12. INITIAL ANOVA FOR CHANGE IN THE PARTIAL PRESSURE OF OXYGEN IN THE MEDIUM OVER THE COURSE OF A PASSAGE IN 850 CM^2 ROLLER BOTTLES INCLUDING FEED TEMPERATURE, ALPHA = 0.05

ANOVA; Var.:O2 (mbar/mio cells); R-sqr=.84666; Adj:.78277 (Design: 2**(5-0) design (Spreadsheet13) in Workbc
5 factors at two levels; MS Residual=.0042243
DV: O2 (mbar/mio cells)

Factor	SS	df	MS	F	p
(1)Aeration	0.02825	1	0.02825	6.6886	0.02382
(2)Cultivation Volume	0.00054	1	0.00054	0.1294	0.72526
(3)Inoculum concentration	0.02588	1	0.02588	6.1275	0.02920
(4)Feed temperature	0.08391	1	0.08391	19.8648	0.00078
(5)Cultivation Temperature	0.00194	1	0.00194	0.4613	0.50988
Error	0.05069	12	0.00422		
Total SS	0.33059	17			

TABLE 13. ANOVA FOR CHANGE IN THE PARTIAL PRESSURE OF OXYGEN IN THE MEDIUM OVER THE COURSE OF A PASSAGE IN 850 CM^2 ROLLER BOTTLES EXCLUDING FEED TEMPERATURE, ALPHA = 0.05

ANOVA; Var.:O2 (mbar/mio cells); R-sqr=.59283; Adj:.46754 (Design: 2**(5-0) design (Spreadsheet13) in Workbc
5 factors at two levels; MS Residual=.0103544
DV: O2 (mbar/mio cells)

Factor	SS	df	MS	F	p
(1)Aeration	0.13056	1	0.13056	12.6100	0.00354
(2)Cultivation Volume	0.01966	1	0.01966	1.8996	0.19138
(3)Inoculum concentration	0.12462	1	0.12462	12.0360	0.00415
(5)Cultivation Temperature	0.04762	1	0.04762	4.5991	0.05146
Error	0.13460	13	0.01035		
Total SS	0.33059	17			

TABLE 14. ANOVA FOR THE CHANGE IN THE PARTIAL PRESSURE OF CARBON DIOXIDE IN THE MEDIUM OVER THE COURSE OF A PASSAGE IN 850 CM^2 ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:CO2 (mbar/mio cells); R-sqr=.95697; Adj:.93904 (Design: 2**(5-0) design (Spreadsheet13) in Workbc
5 factors at two levels; MS Residual=.0004235
DV: CO2 (mbar/mio cells)

Factor	SS	df	MS	F	p
(1)Aeration	0.08114	1	0.08114	191.603	0.00000
(2)Cultivation Volume	0.00298	1	0.00298	7.0519	0.02096
(3)Inoculum concentration	0.01474	1	0.01474	34.825	0.00007
(4)Feed temperature	0.00000	1	0.00000	0.0001	0.99208
(5)Cultivation Temperature	0.01050	1	0.01050	24.808	0.00031
Error	0.00508	12	0.00042		
Total SS	0.11810	17			

TABLE 15. ANOVA FOR TOTAL GLUCOSE CONSUMED OVER THE COURSE OF A PASSAGE IN 850 CM^2 ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:GC (mg); R-sqr=.93318; Adj:.90534 (Design: 2**(5-0) design (Spreadsheet13) in Workbo
5 factors at two levels; MS Residual=6047.283
DV: GC (mg)

Factor	SS	df	MS	F	p
(1)Aeration	20080	1	200805.	33.2058	0.00009
(2)Cultivation Volume	14184	1	141846.	23.4561	0.00040
(3)Inoculum concentration	9596	1	95968.	15.8696	0.00181
(4)Feed temperature	278	1	277.7	0.0459	0.83390
(5)Cultivation Temperature	13137	1	131371.	21.7240	0.00055
Error	72567	12	6047.3		
Total SS	108603	17			

TABLE 16. ANOVA FOR TOTAL LACTATE PRODUCED OVER THE COURSE OF A PASSAGE IN 850 CM^2 ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:LP (mg); R-sqr=.7441; Adj:.63747 (Design: 2**(5-0) design (Spreadsheet13) in Workbo
5 factors at two levels; MS Residual=17867.23
DV: LP (mg)

Factor	SS	df	MS	F	p
(1)Aeration	149500.(	1	149500.(	8.36727	0.01351
(2)Cultivation Volume	132348..	1	132348..	7.40731	0.01855
(3)Inoculum concentration	21003.:	1	21003.:	1.17551	0.29957
(4)Feed temperature	943.8	1	943.8	0.05282	0.82209
(5)Cultivation Temperature	15765.:	1	15765.:	0.88235	0.36608
Error	214406.:	12	17867.:		
Total SS	837844..	17			

8.3 ANOVA FOR EXPERIMENTS IN 1750 CM^2 ROLLER BOTTLES

TABLE 17. ANOVA FOR TOTAL CUMULATIVE CELLS IN SUSPENSION IN 1750 CM^2 ROLLER BOTTLES, ALPHA = 0.05

	ANOVA; Var.:Cumulative cells in suspension; R-sqr=.68975; Adj:.55679 (17 3 factors at two levels; MS Residual=10970E12 DV: Cumulative cells in suspension				
Factor	SS	df	MS	F	p
(1)Aeration	8.859103E+1	1	8.859103E+1	0.80755	0.39870
(2)Media Volume	9.002149E+1	1	9.002149E+1	8.20592	0.02417
(3)Cultivation Temperature	9.832033E+1	1	9.832033E+1	0.08962	0.77334
Error	7.679216E+1	7	1.097031E+1		
Total SS	2.475167E+1	10			

TABLE 18. ANOVA FOR TOTAL VIABLE CELLS HARVESTED AT THE END OF EACH PASSAGE IN 1750 CM^2 ROLLER BOTTLES, ALPHA = 0.05

	ANOVA; Var.:Viable Cell Conc. (cells/ml); R-sqr=.3474; Adj:.06771 (17 3 factors at two levels; MS Residual=227110E7 DV: Viable Cell Conc. (cells/ml)				
Factor	SS	df	MS	F	p
(1)Aeration	4.267267E+1	1	4.267267E+1	0.01878	0.89483
(2)Media Volume	4.009020E+1	1	4.009020E+1	1.76523	0.22564
(3)Cultivation Temperature	3.579035E+1	1	3.579035E+1	1.57590	0.24963
Error	1.589769E+1	7	2.271098E+1		
Total SS	2.436052E+1	10			

TABLE 19. ANOVA FOR VIABILITY OF CELLS AT END OF PASSAGE IN 1750 CM^2 ROLLER BOTTLES, ALPHA = 0.05

	ANOVA; Var.:Viability (%); R-sqr=.32723; Adj:.0389 (175 3 factors at two levels; MS Residual=.000115 DV: Viability (%)				
Factor	SS	df	MS	F	p
(1)Aeration	0.00002	1	0.00002	0.17543	0.68787
(2)Media Volume	0.00008	1	0.00008	0.76698	0.41019
(3)Cultivation Temperature	0.00011	1	0.00011	1.00940	0.34850
Error	0.00080	7	0.00011		
Total SS	0.00119	10			

TABLE 20. ANOVA FOR THE POPULATION DOUBLING LEVEL AT THE END OF EACH PASSAGE IN 1750 CM² ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:Specific NDP; R-sqr=.12281; Adj:0. (175
3 factors at two levels; MS Residual=.2023192
DV: Specific NDP

Factor	SS	df	MS	F	p
(1)Aeration	0.03043	1	0.03043	0.15040	0.70967
(2)Media Volume	0.11698	1	0.11698	0.57823	0.47183
(3)Cultivation Temperature	0.00812	1	0.00812	0.04016	0.84686
Error	1.41623	7	0.20231		
Total SS	1.61450	10			

TABLE 21. ANOVA FOR THE CHANGE IN THE PH OF THE MEDIUM OVER THE COURSE OF A PASSAGE IN 1750 CM² ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:delta pH; R-sqr=.62966; Adj:.47095 (175
3 factors at two levels; MS Residual=.0000251
DV: delta pH

Factor	SS	df	MS	F	p
(1)Aeration	0.00011	1	0.00011	4.51807	0.07113
(2)Media Volume	0.00002	1	0.00002	0.93217	0.36646
(3)Cultivation Temperature	0.00005	1	0.00005	2.32806	0.17089
Error	0.00017	7	0.00002		
Total SS	0.00047	10			

TABLE 22. ANOVA FOR THE CHANGE IN THE PARTIAL PRESSURE OF CARBON DIOXIDE IN THE MEDIUM OVER THE COURSE OF A PASSAGE IN 1750 CM² ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:delta CO2; R-sqr=.93989; Adj:.91412 (175
3 factors at two levels; MS Residual=247.3709
DV: delta CO2

Factor	SS	df	MS	F	p
(1)Aeration	21130.1	1	21130.1	85.4190	0.00003
(2)Media Volume	91.88	1	91.88	0.3714	0.56147
(3)Cultivation Temperature	605.94	1	605.94	2.4495	0.16154
Error	1731.6	7	247.37		
Total SS	28804.9	10			

TABLE 23. ANOVA FOR THE CHANGE IN THE PARTIAL PRESSURE IF OXYGEN IN THE MEDIUM OVER THE COURSE OF A PASSAGE IN 1750 CM^2 ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:delta O2; R-sqr=.09803; Adj:0. (175
3 factors at two levels; MS Residual=5364.313
DV: delta O2

Factor	SS	df	MS	F	p
(1)Aeration	190.4	1	190.41	0.03549	0.85590
(2)Media Volume	65.37	1	65.37	0.01218	0.91519
(3)Cultivation Temperature	3591.0	1	3591.07	0.66943	0.44020
Error	37550.1	7	5364.31		
Total SS	41631.1	10			

TABLE 24. ANOVA FOR TOTAL GLUCOSE CONSUMED OVER THE COURSE OF A PASSAGE IN 1750 CM^2 ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:Total Glucose Consumed per passage (g); R-sqr=.91028; Adj:.87183 (17
3 factors at two levels; MS Residual=.0257283
DV: Total Glucose Consumed per passage (g)

Factor	SS	df	MS	F	p
(1)Aeration	0.54737	1	0.54737	21.2753	0.00244
(2)Media Volume	0.30978	1	0.30978	12.0405	0.01040
(3)Cultivation Temperature	0.06776	1	0.06776	2.6337	0.14864
Error	0.18009	7	0.02572		
Total SS	2.00729	10			

TABLE 25. ANOVA FOR TOTAL LACTATE PRODUCED OVER THE COURSE OF A PASSAGE IN 1750 CM^2 ROLLER BOTTLES, ALPHA = 0.05

ANOVA; Var.:Total Lactate Produced per passage (g); R-sqr=.66891; Adj:.52702 (17
3 factors at two levels; MS Residual=.0856341
DV: Total Lactate Produced per passage (g)

Factor	SS	df	MS	F	p
(1)Aeration	0.14786	1	0.14786	1.72675	0.23024
(2)Media Volume	0.48896	1	0.48896	5.70992	0.04820
(3)Cultivation Temperature	0.00190	1	0.00190	0.02226	0.88559
Error	0.59943	7	0.08563		
Total SS	1.81050	10			

TABLE 26. ANOVA FOR THE RATIO OF LACTATE PRODUCTION TO GLUCOSE CONSUMPTION OVER THE COURSE OF A PASSAGE IN 1750 CM^2 ROLLER BOTTLES, ALPHA = 0.05

	ANOVA; Var.:L/G ratio; R-sqr=.24731; Adj:0. (1750 3 factors at two levels; MS Residual=.0563779 DV: L/G ratio				
Factor	SS	df	MS	F	p
(1)Aeration	0.03471	1	0.03471	0.61582	0.45833
(2)Media Volume	0.03379	1	0.03379	0.59945	0.46413
(3)Cultivation Temperature	0.01899	1	0.01899	0.33687	0.57983
Error	0.39464	7	0.05637		
Total SS	0.52431	10			

8.4 ANOVA FOR INITIAL PERFUSION RATE

TABLE 27. ANOVA FOR EFFECT OF INITAL PERFUSION PARAMETERS ON Z-NUMBER, ALPHA = 0.1

ANOVA; Var.:Z number; R-sqr=.97868; Adj:.92539 (Design: 2**(4-0) design (Spreadsheet2) in Workbo
4 factors at two levels; MS Residual=3.99322
DV: Z number

Factor	SS	df	MS	F	p
(1)Initial	44.597	1	44.597	11.1683	0.07906
(2)Increase	6.2807	1	6.2807	1.5728	0.33650
(3)Rate	22.335	1	22.335	5.5933	0.14174
(4)Increase type	213.606	1	213.606	53.4923	0.01818
1 by 3	31.425	1	31.425	7.8695	0.10705
Error	7.9864	2	3.9932		
Total SS	374.644	7			

TABLE 28. SPECIFIC ANOVA FOR EFFECT OF INITAL PERFUSION RATE AND INCREASE TYPE ON Z-NUMBER, ALPHA = 0.1

ANOVA; Var.:Z number; R-sqr=.7368; Adj:.63151 (Design: 2**(4-0) design (Spreadsheet2) in Workbo
4 factors at two levels; MS Residual=19.72156
DV: Z number

Factor	SS	df	MS	F	p
(1)Initial	210.084	1	210.084	10.6525	0.02235
(4)Increase type	144.210	1	144.210	7.3123	0.04257
Error	98.607	5	19.721		
Total SS	374.644	7			

8.5 CORRELATION COEFFICIENTS FOR ALL VARIABLES AT EACH HARVEST THAT WAS ANALYSED FOR Z-NUMBER

TABLE 29. CORRELATION COEFFICIENTS FOR ALL VARIABLES AT EACH HARVEST THAT WAS ANALYSED FOR Z-NUMBER PART I

	Spearman Rank Order Correlations (Spreadsheet2) MD pairwise deleted Marked correlations are significant at p <.05000							
Variable	q [Gluc]	q [Lac]	Glutamine per cell	Lactate per cell	Neutrals	Tri-	Tetra-syalilated	Corrected Z number
q [Gln]	-0.23789	0.36454	0.17767	-0.27485	-0.03696	-0.04296	0.02176	0.06625
q [Glu]	-0.39868	0.48180	0.12307	-0.24484	-0.20506	-0.20412	-0.05328	0.11186
q [Gluc]	1.00000	-0.83377	0.34896	0.76791	-0.37673	0.21801	0.24400	0.32790
q [Lac]	-0.83377	1.00000	-0.26904	-0.92420	0.23921	-0.25253	-0.13293	-0.22739
q [NH4+]	0.26735	-0.48011	-0.26135	0.39531	0.08311	0.12514	-0.01397	-0.06531
q [Na+]	-0.74277	0.66885	-0.29455	-0.61707	0.32439	-0.04258	-0.07570	-0.25723
q [K+]	-0.61894	0.67842	0.02945	-0.46303	0.06322	-0.25591	-0.18105	-0.09018
Glucoseper cell	-0.63039	0.67748	0.33414	-0.46097	-0.01031	-0.09606	-0.03011	0.04955
Glutamine per cell	0.34896	-0.26904	1.00000	0.45422	-0.46941	0.19249	0.24851	0.45065
Ammonium per cell	-0.59868	0.76191	0.22889	-0.57260	0.02626	-0.15328	-0.01660	-0.00760
Lactate per cell	0.76791	-0.92420	0.45422	1.00000	-0.36510	0.31125	0.18884	0.37633
Poids de la récolte (kg)	0.60875	-0.69871	-0.19503	0.43107	-0.06154	0.07430	0.00684	0.00783
r-hLH IFMA	-0.11558	0.29599	0.14401	-0.29008	-0.03349	0.13519	0.29239	0.11258
Quantité totale de r-hLH dans la récolte (g)	0.51538	-0.52082	-0.13095	0.24296	0.12232	0.23921	0.20385	-0.03547
Production cumulée (g)	0.66716	-0.79962	-0.14352	0.63752	0.01876	0.23996	0.06238	0.02505
Protéines totales LOWRY mg/ml	0.13925	-0.17359	-0.13287	0.06211	0.03762	0.09458	0.10397	0.01572
Quantité totale de protéines dans la récolt mg	0.48255	-0.61163	-0.18874	0.36923	-0.00018	0.21894	0.13509	0.05096
MASS RATIO	-0.17673	0.37467	0.27185	-0.26885	-0.05084	-0.04015	0.09447	0.03059
Neutrals	-0.37673	0.23921	-0.46941	-0.36510	1.00000	-0.21594	-0.45199	-0.85796
Mono-	0.20555	-0.10479	0.28933	0.11764	-0.49939	-0.49348	-0.26260	0.12389
Di-	0.11594	-0.00769	0.29606	0.09849	-0.56247	-0.56435	-0.38313	0.15691
Tri-	0.21801	-0.25253	0.19249	0.31125	-0.21594	1.00000	0.86383	0.62962
Tetra-syalilated	0.24400	-0.13293	0.24851	0.18884	-0.45199	0.86383	1.00000	0.75537
Corrected Z number	0.32790	-0.22739	0.45065	0.37633	-0.85796	0.62962	0.75537	1.00000

TABLE 30. CORRELATION COEFFICIENTS FOR ALL VARIABLES AT EACH HARVEST THAT WAS ANALYSED FOR Z-NUMBER PART II

Variable	Spearman Rank Order Correlations (Spreadsheet2) MD pairwise deleted Marked correlations are significant at p <.05000 Specific perfusion rate	q [G] before	q [G] after	Gln	Corrected Z number
PD	0.02850	0.70974	0.67671	0.65649	0.09470
Débit L/24h	0.16885	0.59268	0.58949	0.60424	0.02543
Taux de dilution	0.16885	0.59268	0.58949	0.60424	0.02543
Change in perfusion rate per day	-0.08198	0.19099	0.17917	0.24488	0.01323
Change in perfusion rate per harvest	-0.08198	0.19099	0.17917	0.24488	0.01323
Specific perfusion rate	1.00000	0.55384	0.59849	0.41649	0.41218
Change in specific perfusion rate per day	0.19624	0.03940	0.05553	0.23043	-0.19980
Change in specific perfusion rate per harvest	0.19624	0.03940	0.05553	0.23043	-0.19980
pH-mètre externe	0.08620	0.17617	0.19214	-0.00352	0.11541
Conso NaOH (g/24h)	0.20957	0.78230	0.77405	0.66549	0.12407
Cumul conso NaOH (g)	0.33208	0.89193	0.88574	0.76224	0.29280
Conso Antimousse (g/24h)	-0.17752	-0.03370	-0.03454	0.06056	-0.12002
Cumul conso Antimousse (g)	-0.03883	0.65947	0.62514	0.59298	0.01755
Cellules totales en suspension (x10.^{6})/ml	-0.20321	0.26720	0.23455	0.35316	-0.08799
Cellules vivantes en suspension (x10.6)/ml	-0.21501	0.21802	0.18030	0.32484	-0.05105
Viabilité %	-0.30675	-0.15966	-0.20863	-0.11812	0.09600
Cellules totales sur microporteurs (x10.6)/ml	-0.47593	0.12430	0.08903	0.24645	-0.27739
Cellules vivantes sur microporteurs (x10.6)/ml	-0.43099	0.14851	0.11989	0.26893	-0.25580
Viabilité %	0.25009	-0.09230	-0.06660	-0.09673	0.30453
Total cellules viables (x10. ^{6})/ml	-0.44484	0.17016	0.13602	0.28579	-0.25695
Glucose before	-0.01454	-0.79347	-0.75725	-0.65625	-0.12764
Lactate before	0.08782	0.80362	0.77547	0.74225	0.11254
Glucose after	-0.01294	-0.79249	-0.75628	-0.65631	-0.12556
Lactate after	0.08697	0.80277	0.77472	0.74056	0.11170
q [G] before	0.55384	1.00000	0.99531	0.78466	0.33513
q [L] before	-0.41857	-0.93320	-0.92532	-0.83177	-0.27779
q [G] after	0.59849	0.99531	1.00000	0.78401	0.33532
q [L] after	-0.44352	-0.94334	-0.93677	-0.82904	-0.30144
Ratio before	0.16078	0.71219	0.69812	0.74713	0.12078
Ratio after	0.17448	0.74127	0.72589	0.76581	0.13917
Dosage r-hLH IFMA µg/ml	-0.04456	-0.13519	-0.14100	-0.14933	0.11258
Productivité volumétrique IFMA µg/ml de bio./jour	0.08930	0.54859	0.54521	0.46303	-0.07226
Productivité spécifique IFMA µg/Mio cells/jour	0.65422	0.36097	0.38649	0.24610	0.28539
OSMO	0.09792	0.46746	0.47666	0.43473	0.07317
pCO2	0.21451	0.20935	0.21817	0.20005	0.10636
pO2	-0.16349	-0.02854	-0.04920	-0.02296	0.07121
Gln	0.41649	0.78466	0.78401	1.00000	0.33979
Glu	-0.09245	0.40386	0.38388	0.40821	-0.10443
Gluc	0.04775	-0.68727	-0.65134	-0.58297	-0.12505
Lac	0.08218	0.75350	0.72874	0.72287	0.13538
NH4+	-0.34154	-0.85432	-0.84588	-0.87987	-0.26100
Na+	0.21923	0.60117	0.60785	0.44951	0.18183
K+	-0.14377	0.46032	0.42419	0.41272	-0.02028
Corrected Z number	0.41218	0.33513	0.33532	0.33979	1.00000