Carbon Dioxide Control in Bioreactors and the Application of Principal Component Analysis to Cell Culture Process Data

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Abstract

Carbon dioxide levels can vary with bioreactor scale in mammalian cell cultures but its effects on cell culture process is poorly understood. Varied results have been presented in the literature on the effects of pCO_2 on the process performance of mammalian cell cultures. If pCO_2 control can be demonstrated to show that it has the potential to effect or improve cell culture performance then the control at production scale and during scale-up would be beneficial.

The primary objective of the research was to examine the effect of carbon dioxide concentration (pCO_2) on a monoclonal antibody (mAb) producing Chinese Hamster Ovary (CHO) cell line in order to assess if pCO_2 is a critical process parameter. During the research investigations, increasing pCO_2 control resulted in an associated increase in osmolality; therefore, the role of the osmolality increase was also investigated. The main process responses examined were the productivity and product quality of a cell culture process, as well as the effect of pCO_2 on the cell metabolism. The effect of pCO_2 control from low to high (20 to 100 mmHg) and at elevated levels (140 mmHg) was examined and compared to a baseline condition (no pCO_2 control).

Results indicated that both increasing pCO_2 and osmolality level independently led to increased product titre. Operation at low to high pCO_2 gave reduced product quality attributes, compared to the baseline. Elevated pCO_2 control resulted in equivalent product quality attributes compared to the baseline. Carbon dioxide level had the greatest impact on the product quality attributes, with osmolality playing little or no role. In summary, pCO_2 control level was shown to be a critical process parameter and should be considered in quality by design, small scale optimisation and scale-up studies.

The current research results disagreed with a selection of the varied results reported in the literature, on the effect on pCO_2/osmolality on a mammalian cell culture process. In previous studies non-optimised process controls and conditions were used, such as the use of unrepresentative reactor vessels (e.g. T-flasks). This would lead to interacting effects from other process parameters. The majority of studies of studies on the effects of pCO_2/osmolality carried out were conducted over 10 years ago. Since then, advances in process monitoring and control, media formulation, cell lines, analytical techniques and large increases in product titres, have occurred.

Principal component analysis (PCA) was applied, with the aim of extracting further understanding from the data set generated in the pCO_2 study. PCA models demonstrated grouping of pCO_2 control and osmolality levels due to differences in process variables, relating to the control of pCO_2. Overall, the PCA results statistically confirmed that pCO_2 control leads to operational differences in the cell culture performance.

Acknowledgements

Contents

i. Nomenclature List

Acronym	Meaning
ATP	Adenosine Triphosphate
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
CQA	Critical Quality Attribute
CPP	Critical Process Parameter
cGMPs	Current Good Manufacturing Practices
CHO	Chinese Hamster Ovary
CIP	Clean in Place
Cys	Cysteine
DO	Dissolved Oxygen
DoE	Design of Experiments
FDA	Food and Drugs Administration
Gln	Glutamine
Glu	Glutamate
Glx	L-alanine-L-glutamine (Ultraglutamine)
Gly	Glycine
His	Histidine
Ile	Isoleucine
kLa	Mass Transfer Coefficient
Leu	Leucine
Lys	Lysine
mAb	Monoclonal Antibody
MBR	Microbioreactor
Met	Methionine
MIR	Mid-infrared
MVDA	Multivariate Data Analysis
Na^+	Sodium
NH_4^+	Ammonium

NIR	Near-infrared
PAT	Process Analytical Technologies
PCA	Principal Component Analysis
PC	Principal Component
pCO$_2$	Partial Pressure Carbon Dioxide
Phe	Phenylalanine
PLS	Partial Least Squares
Pro	Proline
PTM	Posttranslational Modification
QbD	Quality by Design
Qp	Specific Productivity
Ser	Serine
SIP	Steam in Place
STR	Stirred Tank Reactor
TCA	Tricarboxylic Acid Cycle
tPA	Tissue plasminogen activator
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

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1 Introduction and Background Research in Mammalian Cell Culture

1.1 Introduction

The production of recombinant proteins, such as monoclonal antibodies (mAbs), is a multi-billion dollar industry, with global sales of over $125 billion per year predicted for mAbs alone by 2020. (Ecker *et al*, 2015). Monoclonal antibodies have become increasingly important in the biopharmaceutical industry, representing more than 30% of total biopharmaceutical productions with over $20 billion sales in the US alone in 2011 (Bandaranayake and Almo, 2014; Rodrigues *et al*, 2009). The majority of approved mAbs, for use in therapeutic applications, are produced by mammalian cells.

The Chinese hamster ovary (CHO) cell line is a commonly used mammalian production host for mAbs. Chinese hamster ovary cell lines are the work horse of the industry and are well-studied, making regulatory aspects simpler (Rodrigues *et al*, 2009). Mammalian cell lines are favoured over microbial cell lines due to their ability to carry out post-translational modifications (PTMs) of proteins required for human use. Mammalian cells, however, suffer from slow growth rates, high nutrient consumption rates and accumulation of toxic metabolites which are associated with the environmental, chemical and biological factors of the production process. These factors can result in low cell growth rates, productivity and reduced product quality. Therefore, the monitoring and control of these factors is essential to improve productivity whilst maintaining/improving product quality. These factors need to be optimised and considered during scale-up and process development and scale up.

Typically, process, cell line and media development are carried out in shake flasks, which lack the control capabilities and comparability of large scale vessels. Nevertheless, small scale studies are essential to gain information on larger scale production due to the time and costs associated with larger scale runs. Some small scale studies make use of bench top STRs to gain information for process development before scaling up into larger scale vessels. Due mainly to mass transfer limitations, gassing and mixing, performance differences occur between the small and large scale vessels. Much effort has been conducted in the literature, examining the biological, chemical, environmental and mechanical parameters which effect cell culture systems and the scalability. A summary of some of the parameters examined are shown in Table 1.1.

Process Parameters
Temperature
pH
Dissolved Oxygen (DO)
Carbon Dioxide Partial Pressure (pCO$_2$)
Osmolality
Agitation/Mixing
Gassing
Nutrient/Metabolites (glucose, glutamine etc.)

Table 1.1 - Example Cell Culture Process Parameters Investigated in Literature

One parameter, of which its effects on cell culture is poorly understood, is the level of carbon dioxide partial pressure (pCO$_2$). Carbon dioxide levels vary with scale; with a build-up above physiological levels (>70 mmHg) at the larger scale and lower levels at the small scale. This is mainly due the lack of consideration of pCO$_2$ in small scale studies and a build-up at large scale from poor pH control and conservative agitation/aeration rates due to a perceived concern of shear (Nienow, 2015). Carbon dioxide has been shown in the literature to affect the productivity and product quality of cell culture processes. There is a lack of consideration for the control or effect of pCO$_2$ on cell culture, especially during small scale studies. If pCO$_2$ control can be demonstrated to show that it has the potential to effect or improve cell culture performance then the use at production scale would be beneficial.

Therefore, the current research has focused on investigating the effects of pCO$_2$ levels on a mammalian cell culture. Additionally, the data generated has been examined using the multivariate data analysis tool; principal component analysis (PCA). The overall research aim has been to determine if pCO$_2$ control level affects a mammalian cell culture process and to define if it is a critical process parameter. A detailed account of the nature and the objectives of the research undertaken in this thesis is included in Chapter 1.4 (Research Objective). Prior to undertaking the study into the effects of pCO$_2$ control level, a literature review of the use of mammalian cells in the biopharmaceutical industry and the effects of carbon dioxide (CO$_2$) on cell culture processes has been undertaken. A summary of the literature review on the subject is presented below.

1.2 Review of Research on Mammalian Cell Culture

The first therapeutic protein produced from recombinant mammalian cells to obtain market approval was human tissue plasminogen activator in 1986 (Wurm, 2004). By 2004, 60-70% of all recombinant proteins were produced using mammalian cells, with many more in development. The majority of these proteins were produced in suspension cultures, with Chinese hamster ovary (CHO) cells being the most popular cell line choice. Increasing numbers of blockbuster drugs have now

been produced in mammalian cell lines with over 100 recombinant protein therapeutics approved by the US Food and Drug Administration (FDA) to date (Bandaranayake and Almo, 2014).

Microbial cell lines are attractive over mammalian cell lines as they are fast growing, highly productive and low cost, whilst having no virus concerns for regulatory authorities. However, many biopharmaceuticals are too large and complex for microbial cell lines. Complex biomolecules such as mAbs are highly glycosylated proteins, which require post-transcriptional metabolic machinery only available in mammalian cells (Zhang, 2010).

Post-translational modifications, such as glycosylation, of mAbs are important for their biological function and pharmacokinetics. Correct post-translational modifications to proteins as observed in mammalian cells convey higher quality and efficacy, compared to those produced by microbes (Hossler *et al*, 2009; Zhang, 2010).

Mammalian cells are the preferred production host for most complex protein therapeutics, as their functionality and pharmacokinetically relevant post-translational modification are highly human compatible. Despite the challenges compared to microbial systems, including lower yields, higher manufacturing costs and cells fragility, mammalian cell lines have become the most commonly used platform for producing recombinant proteins (Li *et al*, 2005; Zhang, 2010).

1.2.1 Chinese Hamster Ovary Cells

The mammalian Chinese hamster ovary cell line is the work horse of the pharmaceutical industry. Due to the widespread adoption of this cell line, their growth characteristics, cell metabolism, behaviour in bioreactors, virulence factor and likely host cell-related impurities are relatively well understood. Because of the strong regulatory history, more and more products are in development for production in CHO cells, such as monoclonal antibodies (Li *et al*, 2005; Zhang, 2010).

1.2.2 Monoclonal Antibodies

Recombinant protein production is a multi-billion dollar sector of the biopharmaceutical industry, with global sales exceeding $120 billion per year and predicted to grow by $30 billion by 2015 (Bandaranayake and Almo, 2014). As shown in Figure 1.1, of the top nine types of biologic drugs sold in 2011, mAbs have the greatest sales. Monoclonal antibodies are being increasingly used in therapeutics because of their high specificity, low toxicity, long half-life, predictable pharmacokinetics and high dosage demands (Li *et al*, 2005; Zhang, 2010). Currently, mAbs play an important role in the treatment of cancers and autoimmune and inflammatory diseases (Zhang, 2010).
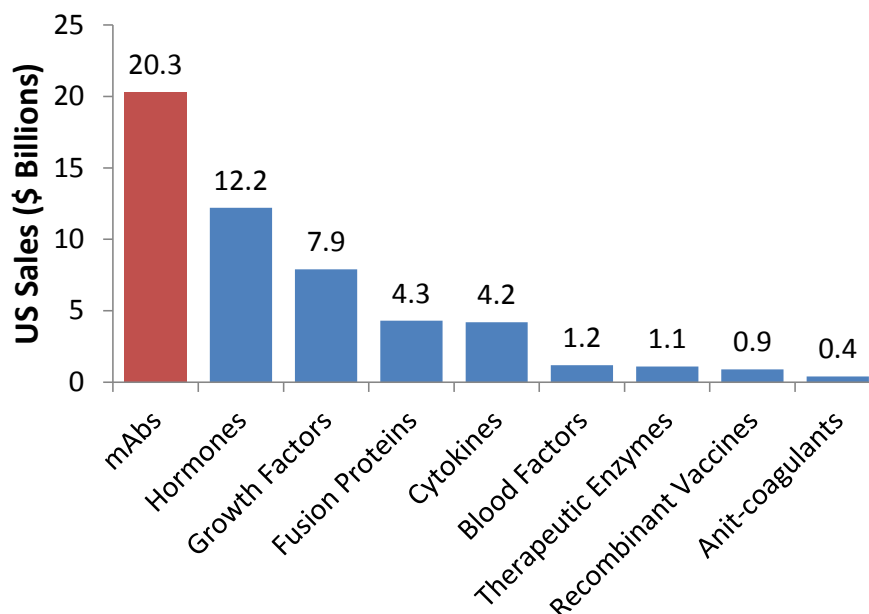
Figure 1.1 - US Sales of the Top Nine Classes of Biologic Drugs in 2011 (adapted from Bandaranayake and Almo, 2014)

Monoclonal Antibodies Productivity and Product Quality

MAb productivity is a function of the cell specific productivity and the viable cell concentration, which are both influenced by a number of cell culture conditions. As cell growth and product formation are generally separate processes in mammalian cell cultures, a balance between the two processes is required (Altamirano *et al*, 2001). Traditionally, most effort has been focused on the optimisation of processes to increase overall productivity; however, more important is the product quality attributes of a mAb as it can affect the safety and efficacy of the drug product.

Product quality is an essential consideration in the biopharmaceutical industry, particularly with the adoption of the Quality by Design (QbD) and Process Analytical Technology (PAT) initiatives. The identification and control of Critical Quality Attributes (CQAs), such as product quality attributes, for monoclonal antibodies is debatably the most challenging step in the application of QbD for development and production of biopharmaceuticals (Rathore *et al*, 2014).

Glycosylation and Charge Variants (Product Quality Attributes)

Monoclonal antibodies are complex biomolecules, which are subject to a number of post-translation modifications and contaminants (Table 1.2). The product quality and efficacy of recombinant antibodies stem from their biological activity and specificity to target antigens, a molecule that triggers the production of an antibody in the body.

Product Variants	Purity (including process-related impurities)
Aggregation	Cell Culture Medium Components
Conformation	DNA
C-terminal lysine	HCP (Host Cell Protein)
Deamided isoforms	Microbiological Purity
Disulfide bonds	Protein A
Fragmentation	Purification Buffer Components
Glycation	Selective agent
Glycosylation	Viral Purity
Oxidation	
Thioether link	

Table 1.2 - Typical Quality Attributes for a Monoclonal Antibody (Adapted from CMC Biotech Working Group, 2009)

The efficacy of an antibody therapeutic is critically dependant on appropriate post-translational modifications (PTM), such as glycosylation. Glycosylation is "a co-translational PTM that results in the attachment of a glucosylated high mannose oligosaccharide, which is trimmed to a specific structure that is bound by chaperones that aid and monitor folding fidelity" (Jefferis, 2005).

Monoclonal antibody charge variant analysis provides the relative composition of acidic, basic and target (unmodified) mAb of the total mAb. The target mAb is the desired mAb and the enzymatic processes, spontaneous degradation and modifications can lead to the formation of acidic and basic variants, which can affect the safety and efficacy of the drug product. As a result of the potential influence of charge variants on the stability, efficacy and safety of mAb, they have gained significant attention (Khawli *et al,* 2010).

Monoclonal antibodies and complicated glycoproteins are critically affected by the glycosylation processes and other protein modifications, as listed in Table 1.2 (Hossler *et al,* 2009; Li *et al*, 2005). During the production of therapeutic proteins (such as mAbs), glycosylation is a significant issue because deviations in micro- or macro-heterogeneity can change product properties and efficacy (Zanghi *et al*, 1999). Glycosylation attributes of glycoproteins can affect many of their properties, such as solubility, activity, circulatory half-life and immunotolerance (Zanghi *et al*, 1999).

Studies have shown that CHO cells are able to produce antibodies with major glycoforms identical to glycoforms present in human antibodies. However, under non-optimal conditions, CHO cells can produce a number of abnormally glycosylated antibodies (products) that lack effectiveness (Jefferis, 2005; Jenkins *et al*, 1996). Whilst optimising process conditions to achieve high productivity, it is critical to monitor product quality changes at every stage of development, as a number of process factors affect the product quality attributes of mAbs (Li *et al*, 2005).

Although a number of factors affect the quality and efficacy of a protein, glycosylation is the main indicator used in many studies in order to assess product quality, as it affects the stability and folding of the protein.

The effects of cell culture media and culture conditions on antibody glycosylation have been extensively studied. Temperature, dissolved oxygen (DO), carbon dioxide, nutrient and metabolite concentrations and culture osmolality have been shown to affect glycosylation (Butler, 2006; Clark *et al*, 2004; Hossler *et al*, 2009; Jefferis, 2005; Jenkins *et al*, 2006). During the production of specific recombinant proteins it is vital that a constant glycosylation profile is maintained from batch to batch. Therefore, in order to control the glycosylation profile, it is important to define/understand the relationship between culture conditions and the effect on glycosylation (Butler, 2006). Not only are there cost implications associated with the protein quality, from reduced product efficacy, but also regulatory concerns over batch-to-batch consistency due to its use in humans (Hossler *et al*, 2009).

1.2.3 Mammalian Cell Metabolism

Lactate and Ammonium – Key Metabolites

Mammalian CHO cell cultures have high nutrient consumption rates, resulting in nutrient depletion and the accumulation of toxic metabolites, lowering cell viability, productivity and product quality. It is important to understand cell metabolism as a function of culture conditions and the effect on productivity and protein quality in order to optimise cell culture processes (Sengupta *et al*, 2011).

The main carbon and energy source in CHO cell cultures are glucose and glutamine. Glucose and glutamine are converted in the cell to intermediates which aid in the generation of energy (via ATP) and for the formation of cell products. The two substrates are used differently in oxidative phosphorylation and to fuel the tricarboxylic acid (TCA) cycle, which are responsible for the production of energy in the cell (Stryer, 1997). The TCA cycle is a series of chemical reactions which makes use of a number of nutrients and amino acids to generate energy and amino acids precursors for the cell.

Glucose is initially converted to pyruvate (via a number of intermediates) by the process of glycolysis and the pentose phosphate pathway. Under aerobic conditions pyruvate enters the TCA cycle and is oxidised to CO_2 and H_2O to produce energy (ATP). Under anaerobic conditions pyruvate is converted to lactate.

Due to the high flux of glucose/glutamine to pyruvate and the comparatively inefficient coupling between glucose glycolysis and the TCA cycle (i.e. the conversion of glucose to pyruvate and its subsequent use in the TCA cycle to produce ATP), the majority of pyruvate is converted into lactate, leading to a build-up in the culture (Dean and Reddy, 2013; Li *et al*, 2012; Tsao *et al*, 2005; Zagari *et al*, 2013). Lactate build-up leads to the acidification of the culture environment and it is possible that lactate is toxic to mammalian cells (Lao and Toth, 1997; Tsao *et al*, 2005). However, a number of studies have shown that lactate can serve as a preferred carbon source, as its presence can limit the formation of other potentially limiting by-products (Dean and Reddy, 2013; Li *et al*, 2012; Zagari *et al*, 2013).

Ammonium is released by cells due to amino acid metabolism, largely from that of glutamine, which is catabolised to ammonium via an intermediate for use in the TCA cycle (Schneider *et al,* 1996; Stryer, 1997). Glutamine is important as a large amount of metabolic energy is derived from its oxidation, rather than glucose (Schneider *et al,* 1996). The inhibitory effect of ammonium appears to play a more important role in cell culture than lactate (Schneider *et al,* 1996).

Ammonium has been shown to inhibit growth and final cell density with the addition of 1 to 5 mM of ammonium salts. The degree of ammonium inhibition, however, is dependent on culture conditions and cell line type (Schneider *et al,* 1996).

The mammalian cell metabolism is a highly complex and multivariate system. The control of process parameters is essentially an indirect method to control the cell metabolism. Due to the highly complex and multivariate nature of the cell metabolism, a large number of factors/parameters can influence the cell culture performance. Therefore, lack of understanding of the process can lead to variations in process performance, resulting in batch-to-batch variations and product loss. To increase process understanding and allow for the introduction of improved process monitoring and control, the FDA introduced the Quality by Design and Process Analytical Technology initiatives. The aims of these initiatives are to encourage a more scientific and risk-based approach to pharmaceutical development.

1.2.4 PAT and QbD

As part of the FDA's "Pharmaceutical Current Good Manufacturing Practices (CGMPs) for the 21st Century" guidelines emerged for the Quality by Design (QbD) and Process Analytical Technology (PAT) initiatives. The purpose of QbD and PAT is to improve the understanding of the pharmaceutical production process, in order to ease regulatory pressure (Abu-Absi *et al*, 2010; Abu-Absi *et al*, 2011; Rathore *et al*, 2010).

Quality by Design

Quality by Design (QbD) is expected to introduce design quality into a process as opposed to testing quality into product. It is a deliberate design effort through the lifecycle of a product, from product conception to process design and finally commercialisation to ensure a better product quality and efficacy (Abu-Absi *et al*, 2010).

The benefits of QbD include the reduction of post-commercial fillings for process changes and a greater flexibility in manufacture due to the creation of a "design space" and increased process knowledge. The creation of a design space for a biopharmaceutical process is important for a robust and well-controlled process. A process design space, defined by the FDA, is "the multi-dimensional combination and interaction of input variables that have been demonstrated to provide an assurance of quality" (Abu-Absi *et al*, 2010).

Creation of a design space involves the determination of Critical Process Parameters (CPPs) that affect the critical quality and performance attributes of a process. Variations, greater than the normal operating ranges, of key process parameters are determined, which aid in determining the acceptable variations in process parameters. This allows more flexibility in manufacturing, without the need to consult regulatory authorities for variations within the design space (Abu-Absi *et al*, 2010).

The QbD approach involves identification of the product attributes that are of significant importance to the product's safety and/or efficacy and the design of the process to deliver these attributes. This allows for the design of a robust control strategy to ensure consistent process performance, validation and filling of the process, demonstrating the effectiveness of the control strategy and finally on-going monitoring to ensure robust process performance over the life cycle of the product (Abu-Absi *et al*, 2010).

The process of characterising a design space is carried out a using a qualified scale-down model (Abu-Absi *et al*, 2010). QbD is essential to attain which CPPs require monitoring and control, hence it is important that these small scale studies are representative of the production scale process.

Microbioreactors

Typically small scale studies, cell line and media development for process optimisation, have been carried out in shake flasks and micro-well plates, which lack the control capabilities and comparability of large scale vessels. Small scale studies are important to gain information on larger scale production reactors, due to the time and costs associated with larger scale runs and are a key

part of QbD. A typical process development study involves iterative rounds of DoE studies, examining a number of parameters. These studies can easily lead to a significant number of experiments, which are not economically viable or of a high-throughput nature at the bench scale or larger volume bioreactors. To overcome these problems, miniaturised production systems, which are able to mimic large scale production and control and offer high-throughput, are desirable. Microbioreactors (MBR) offer potential tools as scale-down models of laboratory-scale bioreactors. MBRs present the ability to generate high throughput experimental data under controlled conditions in an economically viable way (Schapper *et al*, 2009).

MBRs can be classed into two main categories; shaken and stirred systems. Shaken systems, which include the Micro-24, micro-matrix and M2P Biolector, make use of orbital shaken well plates, which have been extensively used historically for cell line selection and high throughput process development. These systems distinguish themselves from well plates with the addition of pH, DO and temperature sensors and control. The differing physical characteristics of mixing and gassing strategies between small scale shaken and large scale stirred systems lead to concerns over potential differences in cell culture performance. Commonly, during process development and clone selection, the performance of clones in shaken systems do not transfer to the larger agitated systems, with the differences in mixing and gassing being one possible cause (Nienow, 2015). For example, orbital shaken reactors are more prone to surface aeration influences. Despite these differences in physical characteristics, Chen *et al* (2009) showed scalable performance between the Micro-24 and 2 L bioreactors for a fed-batch CHO culture. However, in their study the scale-up strategy implemented was not discussed in detail, suggesting that the process operating space between the MBR and STR may have been large.

The most prominent stirred MBR on the market is the ambr®15 (Sartorius) which makes use of a mechanically agitated impeller and gas sparging, designed to mimic the design of large scale bioreactors. The operating volume of the Ambr15 is 10 to 15 mL. Other larger volume bioreactors, 50 to 250 mL, exist with mechanical agitators (Eppendorf, Applikon and Sartorius).

Nienow *et al* (2013) investigated the physical characteristics of the ambr15, demonstrating that a number of physical features were different to larger scale STRs, specifically a reduced Reynolds number (leading to transitional flow as opposed to turbulent flow) and reduced superficial gas velocity from low air flow rates. However, despite the differences between scales, mixing times are similar to the larger scale STRs (due to the reduced Reynolds number) and sufficient k_La values could be achieved. Cell culture runs by Nienow *et al* (2013) demonstrated acceptable performance in comparison to 5 L STRs, whereas shake flask cultures appeared to underperform.

Microbioreactors offer the potential for high-throughput experiments in a more representative system, than the traditionally used shake flasks and microwell plates, to mimic larger scale STRs. An appreciation of the inherent differences in scale is required during small scale studies, but if predictable performance during scale-up can be demonstrated, then the application of MBRs is significantly beneficial over the traditional methods. The application of MBRs is essential to generate the DoE data required as part of QbD, allowing for a significant amount of parameters and interacting effects to be assessed with a reduced number of experiments. A drawback to the operation of MBRs is the small operating volumes, meaning that routine offline sampling is not possible. To overcome this, analytics which are able to use a much reduced sample volume, µL as opposed to mL, or improved inline sensors, such as spectroscopy methods (e.g. Raman), are required.

Process Analytical Technology

A key part of the "Pharmaceutical Current Good Manufacturing Practices (CGMPs)" announced by the FDA is the PAT concept (Rathore *et al*, 2010). Process Analytical Technology (PAT) has been defined as "a system for designing, analysing and controlling manufacturing through timely measurements of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality" (Rathore *et al*, 2010).

The PAT framework's aim is to design and develop a well-understood process that can consistently produce a pre-defined level of quality. In general, for a process to be considered well understood:

1. The critical sources of variation are identified and understood;

2. The process is able to handle variability; and

3. That over a design space, the product-quality attributes can be accurately and reliably predicted.

Online monitoring of chemical and biological parameters is limited due to the lack of reliable online sensors. Parameters such as cell density, major nutrients/metabolites and antibody products are normally measured offline through (daily) sampling. This leads to delays in relating culture parameters and implementing control changes.

The enhancement of recombinant mAb protein production is often a result of combined improvements of cell line, culture medium and culture conditions aided by QbD and PAT. Mammalian cell culture parameters, such as the environmental factors, and the accumulation of

toxic metabolites, such as lactate and ammonium, need to be addressed to improve cell growth, productivity and product quality.

Multivariate Data Analysis (MVDA) – Principal Component Analysis (PCA)

A key aim of QbD and PAT is to increase process knowledge for the design and operation of biopharmaceutical processes. Therefore, MVDA along with DoE have a significant role to play in the implementation of the QbD and PAT initiatives. A multivariate approach is needed for the analysis of the datasets generated from bioprocesses due to the highly complex and multivariate nature of cells (Mercier *et al*, 2014). The application of MVDA techniques, such the Principal Component Analysis (PCA) and Partial Least Squares (PLS), can translate the large amounts of data gathered during bioprocesses into smaller, more comprehendible datasets with the relevant information retained. Because of this reason, MVDA is considered crucial for successful PAT and QbD implementation (Mercier *et al*, 2014). MVDA and DoE are important for increasing process knowledge and highlighting the important parameters in mammalian cell cultures, which affect the process performance.

1.2.5 Mammalian Cell Culture Parameters

A balance between cell growth, productivity and product quality needs to be realised in mammalian cell cultures, as in many cases cell growth and product formation are decoupled (Altamirano, 2001; Hossler *et al*, 2009). To achieve this, monitoring and control of process parameters, such as temperature, pH, osmolality, dissolved oxygen (DO), carbon dioxide (CO_2) and nutrient and metabolite concentrations, are required (Ahn *et al*, 2008; Rodrigues *et al* 2009; Trummer *et al,* 2006). Numerous studies have been carried out previously, examining the effect of a number of parameters on culture performance, such as temperature, pH and DO.

Temperature

Temperature is considered to be a highly critical process parameter, as a shift in a few degrees can lead to lowered cell growth or death; whilst also impacting on product quality (Ahn *et al*, 2008; Chuppa *et al,* 1997*;* Clark *et al*, 2004; Hossler *et al*, 2009; Trummer *et al*, 2006; Zhang, 2010).

The ideal growth temperature for most mammalian cell cultures is 37$^{\mathrm{o}}$C. However, it has been shown that a lower temperature can be beneficial for protein production (Rodrigues *et al*, 2009). At lower culture temperatures, increased productivity has been observed along with increased culture duration (from lower cell growth). A balance between cell growth and productivity is required as product titre is a function of the specific productivity (q_p) and the integral of viable cell concentration

(IVC) (Zhang, 2010). Therefore, the use of two temperature profiles, one for growth and one for protein production, may be beneficial (Hossler *et al*, 2009).

Literature suggests that shifting the operating temperature of mammalian cells results in improved cell viability duration and increased productivity; however, this has often been shown to be cell line specific (Ahn *et al*, 2008; Chuppa *et al,* 1997*;* Clark *et al*, 2004; Hossler *et al*, 2009; Trummer *et al*, 2006). Temperatures as low as 30°C have been shown to improve cell viability and productivity in mammalian cell cultures (Trummer *et al*, 2004).

Trummer *et al* (2006) observed a significant effect of temperature on a CHO process producing a highly glycosylated protein. Decreasing the process temperature from 37°C to 33°C led to a significant decrease in the specific growth rate, whereas reduction to 30°C led to an arrest in cell growth. An increase in temperature to 39°C led to decreased growth rates and reduced cell viability. At lower culture temperature (30°C and 33°C) enhanced specific productivity was maintained until the culture end.

Cell metabolism regarding carbon source and amino acids were also strongly affected by low cultivation temperature. A significant reduction in glucose metabolism (and hence lactate production), as well as reduced glutamine consumption rates, were observed, as noted in other studies (Bollati-Fogolin *et al*, 2005). However, at lower temperatures a reduction in protein sialylation was observed, which is contrary to many other studies which indicated an improved or constant rate of sialylation. However, the protein produced in this study was a highly glycosylated one, therefore the impact of process parameters on the glycosylation may have had a greater effect.

Bollati-Fogolin *et al* (2005) demonstrated that a temperature reduction from 37°C to 33°C in a CHO cell culture increased the production of a recombinant glycoprotein by six-fold, whilst maintaining product quality. Ahn *et al* (2008) also demonstrated that the cumulative and specific production rates of a glycoprotein increased at below 37°C and were highest at 32°C due to higher cell viability in a CHO cell line. Cultivating at 32°C had no negative effects on the quality of protein (below this temperature the quality was reduced).

The investigation carried out by Clark *et al* (2004) did not agree with findings in previously discussed studies (Ahn *et al,* 2008; Bollati-Fogolin *et al*, 2005). The reduction in temperature in a CHO cell culture from 37°C to 34°C over an eight day period had no significant effect on productivity or cell viability. However, in their study the temperature was gradually reduced over the course of the culture, as opposed to culturing at a constant (lower) temperature or using a sudden shift in temperature. The culture temperature was the lowest for only a short period and not for the

entirety of the production phase. Clark *et al* suggested that the effect of lowering the culture temperature on mammalian/CHO cell lines may be cell line specific. It is possible that the reduction in temperature was not sufficient, as other investigations examined temperatures as low as 30°C.

Culture pH

Culture pH is considered a critical parameter in mammalian cell cultures as small variations in pH can affect cell growth, productivity and product quality (Rodrigues *et al*, 2009; Trummer *et al*, 2006). Under changing culture pH, the internal pH of the cell and Golgi apparatus (where some glycosylation processes occur) are likely to change, resulting in a reduction of the activities of the key enzymes associated with PTMs (Butler, 2006).

As with temperature, the optimum pH for growth may not be the same for productivity (Rodrigues *et al*, 2009). Initial cell growth has been shown to favour higher pH values, but higher pH values can increase anabolic metabolism (increasing lactate production). It has been shown to be favourable to decrease the pH of a cell culture during the latter stages of protein production, in order to increase the culture duration (Rodrigues *et al*, 2009).

Cell culture pH is affected by a number of factors, including gas sparging, metabolite/nutrient concentrations and buffering capacity (Rodrigues *et al*, 2009). Most bioreactor processes utilise CO_2 and carbonate base to control culture pH. As shown in equation (1.1), increased CO_2 leads to acidification of culture media, by shifting the equilibrium to the right. Therefore, variations in process performance leads to variations in pH and hence variations in pCO$_2$ levels (as a controller action to modify pH).

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \qquad \text{(1.1)}$$
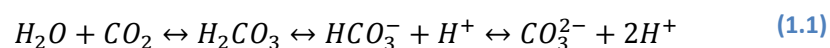
pH is a known critical process parameter and a number of studies have demonstrated the manipulation of culture pH to improve productivity and product quality (Larsson *et al,* 2008; Marasco, 2014; Rendall *et al*, 2005; Trummer *et al*, 2006; Yoon *et al,* 2005). Increased productivity, cell culture duration and reduced lactate production were observed by Rendall *et al* (2005) when the pH of a CHO cell line was reduced below 7.0. Product quality was unaffected by this change in pH. Trummer *et al* (2006) demonstrated that a lower pH of 6.8 increased cultivation duration, resulting in increased glycoprotein production.

Larsson *et al* (2008) also indicated that lowering the culture pH to 6.8 gave a substantially lower lactate production rate whilst allowing a sustained acceptable cell growth in a CHO cell line. Running

the fed-batch cultures constantly at pH 6.8 resulted in significant reduction in lactate levels, compared to a drift in pH from 7.0 to 6.8. However, in their study the effect on product quality was not examined.

These studies indicate that the main benefit of lowering culture pH is the reduction in the cell metabolism, which results in lower levels of toxic metabolites. This suggests that the control of cell metabolism is the most important aspect in these studies, opposed to temperature. Although these studies showed improved cell growth and productivity, the interactions of pH levels with other factors was not examined (i.e. a DoE).

Yoon *et al* (2005) did examine the effect of different levels of pH at two different temperatures (not as a DoE) on glycoprotein excreted by CHO cells. The growth rate was strongly influenced by culture pH and temperature. The culture pH at 32.5^{o}C influenced specific protein production differently than at 37°C. At a temperature of 37°C, the specific productivity was found to be fairly constant in the pH range 6.85-7.80. However, at a lower pH the culture time increased, leading to a greater production of protein product. The lowering of both culture temperature and pH slowed the cell metabolism significantly and the effect of culture pH was more significant at 37°C than at 32.5^{o}C.

The highest specific productivity was observed at pH 7.0 and 32.5^{o}C, compared to other studies (where the pH 6.8 was deemed the optimum). This suggests that the effect of pH is cell line specific or that the interactions between pH and temperature are different at different levels (carrying out a DoE would help determine this relationship). The effect of temperature and culture pH on the glycosylation profile was not examined by Yoon *et a*l.

Dissolved Oxygen (DO)

Oxygen is required by mammalian cells for production of energy from organic matter, which makes dissolved oxygen an important variable in mammalian culture. A number of factors affect the DO level in the medium, including cell growth rate, carbon source and the specific consumption rate of cells. Although DO does not affect growth rate and production in a large range (typically pO$_2$ 20-100%), it can have negative effects on the product quality (glycosylation) at high levels (Li *et al*, 2005; Rodrigues *et al*, 2009). Dissolved oxygen also presents a problem during scale-up as high cell oxygenation needs to be balanced with cell damage (from increased agitation and aeration).

Nutrients/Metabolites

Nutrients present in the culture medium support cell growth and protein production and their depletion can endanger cell growth and product formation. From the numerous nutrients present in

the medium, glucose and glutamine are considered two of the most critical affecting cell growth and protein production (Rodrigues *et al*, 2009).

Consumption of glucose by cells, especially at high concentrations, is carried out at a higher rate than the rate at which it is incorporated into the metabolism cycle (i.e. it is unregulated). This leads to the accumulation of potentially toxic by-products (such as lactate) which can inhibit cell growth, productivity and product quality (glycosylation).

To avoid the build-up of toxic metabolites, attempts have been made by some researches to control the formation of these products, such as the control of glutamine concentration to reduce ammonium formation. The use of fed-batch processes to maintain the concentration of glucose below 2 g.L^{-1} has been used to control the formation of lactate, as well as replacing glucose with galactose (Altamirano *et al*, 2001; Rodrigues *et al*, 2006).

The monitoring and control of nutrient and metabolite concentrations (as well as cell density and viability) are carried out via sampling and analysed using at/off-line methods, which makes the monitoring and control of cell metabolism difficult. The main aim in the pharmaceutical industry is to improve cell culture processes through better monitoring and control. Therefore, inline real time monitoring of these parameters is required.

Probes that offer in-line real time monitoring without sampling, are desirable in order to monitor and control process parameters. Possible solutions include NIR (near-infrared), MIR (mid-infrared) and Raman probes.

NIR and MIR have been widely applied in a number bioprocesses for online monitoring. However, the resultant peaks from NIR are broad and overlapping, making spectra interpretation difficult. Absorbances in the mid-infrared region are stronger and yield more distinct spectral features; however, inference from water can make sample preparation in aqueous systems difficult. It is also difficult to scale NIR and MIR probes and to use them in CIP/SIP systems (Abu-Absi, 2011).

Raman spectroscopy measures the amount of light scatter inelastically at different frequencies by molecular vibrations, which results in very detailed molecular finger prints, with high chemical specificity. Raman probes have been applied much less frequently in the bioprocess (Abu-Absi, 2011).

Raman probes have been applied to monitor offline glucose, glutamine, lactate and ammonium in a CHO cell line culture. Abu-Absi (2011) demonstrated the feasibility of applying a Raman probe to mammalian cell cultures; however, further study is required to the application of this probe for this

purpose. Models produced were fairly accurate for monitoring metabolites and cell growth/viability when compared to the standard deviations for the reference method (offline Nova BioProfile metabolite measurements).

The main drawback with in-line probes, such as NIR, MIR and Raman probe, is the complicated operation, cost and the need for large amounts of historical data for model development. Additionally, cell culture media and metabolism are highly dynamic, with many substances affecting the metabolism pathways and numerous substances able to be converted (irreversible and reversible) to other substances (e.g. pyruvate can be converted reversibly to alanine and lactate). This dynamic nature of the cell makes examining the effect of feed nutrients and waste/by-products difficult, as the feed nutrient can affect a number of pathways.

Osmolality

Osmolality is a measure of the solute concentration of a solution. MAb production, cell growth and death rates are significantly influenced by osmolality, which, in turn, is affected by the addition of base, glucose and other medium concentrates to the culture. However, within a certain range (300-400 mOsm.kg^{-1}); the raised osmolality can be beneficial. In the 300-400 mOsm.kg^{-1} range, this is compensated with increased specific mAb production rate, although a negative effect on cell growth has been shown (Rodrigues *et al*, 2004).

Nutrient, waste and by-product concentrations in the medium affect the culture pH through metabolic activity (i.e. consumption/production). Excess glucose leads to the formation of lactate (through the anaerobic metabolism of pyruvate, which is formed from glucose via glycolysis), which lowers the culture pH. If base addition is required to counteract this process, then the osmolality of the culture will increase, which can be detrimental to cell growth, productivity and product quality (Rodrigues *et al,* 2004). Additionally, metabolites such as glucose and lactate contribute to the media osmolality. Therefore, in batch and fed-batch systems (i.e. non-steady state) osmolality is extremely difficult to control directly.

Seeding Density

Little has been discussed on the effect of seeding density on CHO cells lines producing a monoclonal or glycoprotein product. However, Abu-Absi *et al* (2010) highlighted that the seeding density and cell viability are key factors in a CHO cell culture. A model generated through a DoE study indicated that the seeding density had a significant effect on the cell culture. Generally seeding density is determined by the desired culture time (i.e. higher seeding density for shorter cultures) and available inoculum density achievable (i.e. limited by inoculum vessel size and cell growth rate).

Literature has demonstrated that a number of processing parameters can affect a cell culture process and that differences in the control of these parameters can lead to disparities in performance. In a number of the studies, cell line and process differences led to changes in process responses. This may indicate the need to consider these parameters during the early stages of process development, as they will affect the performance and scale-up of the process. Additionally, the parameters require investigation/consideration in a multivariate nature, as there will be interacting effects due to the complex interconnected nature of the cell metabolism.

An important process parameter which is generally overlooked (due to its use as a controller action) is the pCO_2 level. There is limited literature on pCO_2, with the general understanding that a build-up at large scale is inhibitory to the process performance. A review of literature on pCO_2 control is presented below.

1.3 Carbon Dioxide and Osmolality in Mammalian Cell Culture

Control of pH with sodium carbonate/bicarbonate solution and pCO_2, can lead to an increase in osmolality. As pCO_2 level increase, base solution is added to maintain the pH set point. Salt is a by-product of this reaction, and will accumulate as more base is added. This osmolality change can have an impact on process performance, therefore, when considering pCO_2 control levels, the associated change to osmolality should also be taken into account.

1.3.1 Bioreactor Scale-up and Carbon Dioxide Levels

Physiological partial pressure of CO_2 in cells is generally in the range of 30-70 mmHg. The accumulation of CO_2 (>100 mmHg) in bioreactors, particularly in large scale production vessels, e.g. >500 L, is primarily due to poor mixing and mass transfer coupled with the addition of CO_2 and carbonated buffers to control pH and from cellular respiration.

Small scale bioreactors are unlikely to suffer from the build-up of CO_2 due to the selection of more efficient mixing and aeration parameters, compared to the larger scale bioreactors. This difference in scale can result in a drop in performance, potentially related to differing pCO_2 levels, during the scale-up of a process and leads to inaccurate scale-down models.

The increase in pCO_2 levels with the increase in scale from the bench-top to production STRs has been discussed by a number literature sources (Nienow, 2015; Sieblist *et al*, 2011; Xing *et al*, 2009). Despite there being a significant amount of literature discussing the importance of CO_2 and the stripping of CO_2 from bioreactor systems during scale-up, there are only a small number of

experimental studies examining the effect of pCO_2 levels on cell culture performance (deZengotita *et al*, 1998, deZengotita *et al*, 2002, Gray *et al*, 1996, Kimura and Miller, 1996; Zhu *et al*, 2005).

The build-up of pCO_2 in industrial reactors mainly arises from the addition of CO_2 to control pH, coupled with poor CO_2 removal rates from a reluctance to operate at high airflow rates due to the concern of damage to cells from bubble bursting (Nienow, 2015). CO_2 is much more soluble than oxygen, by more than two orders magnitude, and therefore, gassing intensity has a greater impact on CO_2 removal than agitation speed (Nienow, 2015). For industrial scale reactors the oxygen transfer is achieved with low sparge rates, leading to a reduction in the stripping of CO_2 compared to the smaller scale reactors (Nienow, 2015). This indicates that the issue of pCO_2 build-up is one of process optimisation and performance, highlighting that the factors leading to pCO_2 build-up, such as low gassing and agitation rates and un-optimal control of the culture (e.g. pH), need to be considered during small scale and scale-up studies. Therefore, it can be concluded that carbon dioxide levels differ between bioreactor scales, mainly due to the mode of operation alongside biological factors (i.e. from the control of pH). In addition to the differing overall pCO_2 levels, the time based-profile of pCO_2 concentrations between STR scales also differs, which can lead to differences in cell culture performance (Blombach and Takors, 2015).

Xing *et al* (2009) measured the CO_2 removal rates of a bench-scale and a 5000 L scale bioreactor, demonstrating that CO_2 removal in the 5000 L reactor was 50 % that of the bench-scale, which is likely partially due to a reduction in the achievable power inputs and superficial gas velocities with the increase in scale. Sieblist *et al* (2011) examined the factors effecting CO_2 stripping from industrial reactors. Sieblist *et al* described a method for optimising the control of pCO_2 during scale-up by first setting the gas throughput to a value where pCO_2 is kept in a desired range and then adjusting agitation speed for a desired oxygen transfer rate.

In the studies described above (Nienow, 2015; Sieblist *et al*, 2011; Xing *et al*, 2009), the effect of CO_2 level on cell culture performance was not assessed, particularly for the scale-up of processes. Instead, these studies made reference to a small number of literature sources which used vessels that were not comparable to STR, i.e. static cultures and microwell plates (deZengotita *et al*, 1998, deZengotita *et al*, 2002, Gray *et al*, 1996, Kimura and Miller, 1996; Zhu *et al*, 2005). Additionally in these studies, poor control of pCO_2 levels would have been present, as feedback control of pCO_2 was not applied and instead CO_2 was controlled via environmental CO_2 levels, i.e. the incubator environment CO_2 concentration. Due to the poor mixing and gas exchange/homogeneity in the culture systems used in the pCO_2 level studies, comparability to the large scale STRs is poor.

To the best of the author's knowledge there are no published studies examining the control of pCO_2 at differing levels in a representative cell culture system, i.e. the stirred tank reactors, with the use of feedback pCO_2 control. Despite this, many assertions have been made in literature studies about the detrimental effect of elevated pCO_2 levels in industrial scale reactors, based on a small number of experimental studies.

1.3.2 Carbon Dioxide and Osmolality Studies

The accumulation of carbon dioxide (CO_2) to elevated levels (greater than 100mmHg) has been reported as an issue in industrial scale reactors (Drapeau *et al*, 1990; Ozturk *et al*, 1995; Zanghi *et al*, 1999). However, there are a limited number of studies examining the effect of CO_2, particularly on the productivity and product quality attributes, of a cell culture process (deZengotita *et al*, 1998, deZengotita *et al*, 2002, Gray *et al*, 1996, Kimura and Miller, 1996). Additionally, studies relating to pCO_2 levels in cell culture were carried out over 15 years ago (to the author's knowledge), during which time a number of process improvements have occurred. These advances include changes to media in terms of the removal of serum, which can have interacting effects with other parameters, cell line developments where mAb production has increased from $mg.L^{-1}$ to $g.L^{-1}$ and process monitoring advances with in-line and at-line analytical technologies.

Productivity

Previous studies have shown varying results on the effects of pCO_2 and osmolality on mAb production. A number of studies have stated that elevated osmolality and pCO_2 increase productivity (Ozturk and Palsson, 1990; deZengotita *et al*, 1998; Brady, 2009; Eisenkraetzer *et al*, 2013; Silva *et al*, 2013), whilst other studies have reported that elevated pCO_2 and osmolality have a negative or little impact on cell culture processes (Kimura and Miller, 1996; Drapeau *et al*, 1990; Gray *et al*, 1996).

Gray *et al* (1996) reported that the productivity of a CHO perfusion culture was maximised when pCO_2 was maintained between 30 and 76 mmHg and a dramatic reduction was seen at pCO_2 levels greater than 105mmHg. A 50% decrease in the relative specific productivity of the perfusion culture was reported with an increase in pCO_2 from 35 mmHg to 148 mmHg. The main cause of the decrease in productivity was contributed to the decrease in cell titre and culture viability, from 79% to 58% at 35 and 148 mmHg, respectively. However, the low viability of the control culture (79%) indicates that optimisation of other process conditions is required, as the culture viability of the control perfusion culture was below normally accepted values (95%). Gray *et al* (1996) do not report whether pH was controlled throughout the process, which would also have an effect on the culture at high pCO_2 levels if uncontrolled.

Kimura and Miller (1996) reported that elevated pCO_2 decreased the specific tissue plasminogen activator (tPA) production rate by up to 40% at 250 mmHg compared to 36 mmHg in serum containing media. The starting pH was adjusted to a control value; however, the authors do not mention if pH was controlled throughout the process, which would affect process performance. The study was also carried out in well plates, indicating that other process parameters, which would have an interacting effect with pCO_2/osmolality levels, were not controlled. This is due to the fact that well plates have no monitoring or control capabilities.

deZengotita et al (1998) reported that the differences in specific antibody productivity in well plates were not significant at elevated pCO_2 levels. However, this is likely due to the large variances between repeat runs, indicating other uncontrolled factors may have played a role. The specific antibody production rate calculation is highly sensitive to cell counts and mAb concentrations, determined by haemocytometer and ELISA, respectively, in the study by deZengotita et al (1998). Therefore, inaccuracies in these measurements would lead to large calculated errors. Additionally, there are limited process controls in well plates, which would have an interacting effect on the culture performance, which may be the cause of the differences in results compared to the results presented in this investigation.

Zhu et al (2005) observed that elevated pCO_2 and osmolality had no significant effect on the productivity of a CHO cell line. However, in their study pCO_2 level was used to control pH (i.e. pH and pCO_2 control were not decoupled). Instead bicarbonate was added to maintain the pH at the elevated pCO_2 level, i.e. the equilibrium of pH to pCO_2 was adjusted so that at elevated pCO_2 a similar pH was maintained. The lack of decoupling pH and pCO_2 control would possibly introduce interactions. Zanghi et al (1999) highlighted that the interaction of bicarbonate ions with pH and CO_2 can negatively impact a cell culture process.

A number of studies have reported increased cell specific protein productivity at increased pCO_2/osmolality, although not in the overall titre due to decreased cell growth (Ozturk and Palsson, 1990; deZengotita et al, 1998; Brady, 2009).

Ozturk and Palsson (1990) reported a greater than two-fold increase in specific antibody production rate for a hybridoma culture grown in T-flasks at 435 mOsm compared to 290 mOsm. However, antibody concentrations were similar due to decreased growth rate and increased death rate. Brady (2009) observed a 140% increase in productivity of a monoclonal antibody by a GS-NS0 culture grown at 450 mOsmo.Kg^{-1} compared to those grown at 290 or 320 mOsmo.Kg^{-1}. However, due to lower growth rates the total mAb titre did not increase.

In batch reactors, deZengotita *et al* (1998) demonstrated a 68% increase in specific antibody production rate for hybridoma cells when pCO_2 and osmolality were increased from 36 mmHg and 310 mOsm.Kg^{-1} to 140 mmHg and 370 mOsm.Kg^{-1}. Due to increased NaOH addition to control pH at elevated CO_2, an increase in osmolality was observed. Therefore, it is unclear whether the increase in productivity was due to pCO_2 or osmolality along with other interacting effects. The overall product titre was not disclosed. However, a 14% increase in cell death rate coupled with a 37% decrease in cell growth suggests that a lower overall product titre would have been achieved. The results show that differences in culture vessel can lead to differences in responses, as in the same study little difference was seen at elevated pCO_2 for well plate experiments.

Eisenkraetzer *et al* (2013) concluded that elevated pCO_2 control resulted in a higher productivity and higher product titre. However, the higher product titre was due to a slower decline in cell culture viability and at equivalent time points the uncontrolled batch had a similar titre to the higher pCO_2 condition. Although osmolality was controlled in their study, the uncontrolled low and high pCO_2 conditions displayed differences in osmolality values, of greater than 20 mOsmo.Kg^{-1}. In this work the highest titre was obtained in a dynamic set value profile of 5 to 15% pCO_2, which was designed to mimic pCO_2 enrichment in the culture medium during the fermentation process in industrial production processes.

Sengputa *et al* (2011) highlighted that the carbon dioxide level had a significant effect on the specific protein production of a CHO cell line in a DoE study. However, the effect of osmolality was not discussed, i.e. it is unclear if the osmolality was significantly affected under pCO_2 control.

Product Quality

Little has been published on the effects of elevated pCO_2 and osmolality on the product quality attributes of monoclonal antibodies. Results reported conclude that elevated pCO_2 level has little impact on the product quality attributes of a mAb product (Kimura and Miller, 1997; Schmelzer and Miller, 2002).

Schmelzer and Miller (2002) observed that the macro-heterogeneity of an IgG_2-based mAb was unaffected at elevated values of pCO_2 (up to 250 mmHg) and osmolality. Under elevated osmolality conditions IgG_{2a} occupancy did not change significantly. However, changes in the charge distribution, relating to the isoelectric point, were observed. At a controlled pCO_2 level, of 40 and 195 mmHg, elevated osmolality (435 mOsm.Kg^{-1}) negatively impacted galactose content, 5% and 25%, respectively. Schmelzer and Miller proposed that the reduction in galactosylation was due to an increase in the Golgi apparatus pH.

Zanghi *et al* (1999) showed that elevated pCO_2 can alter protein glycosylation (a product quality attribute) of a recombinant protein, by examining the cell-surface polysialic acid (PSA) content of a protein. However, the decrease in PSA was correlated to the bicarbonate concentration and the associated increase in osmolality (which varied due to the increase in CO_2), indicating that the bicarbonate may play a bigger role than CO_2 on the effect of product quality (Zanghi *et al*, 1999).

Kimura and Miller (1997) reported that elevated pCO_2 levels had little effect on the glycosylation of a recombinant tPA secreted by CHO cells. The percentage of N-glycolylneuraminic acid decreased from 3.2% to 1.9%, as the pCO_2 concentration increased from 36mmHg to 250mmHg. There was no effect on the total sialic acid content, the expression of high mannose oligosaccharides or the distribution of surface charges. These product quality attributes can affect the efficacy and safety of a drug product.

Cell Growth

A number of studies have shown that cell growth is inhibited under elevated osmolality and pCO_2. Kimura and Miller (1996) noted a 15% and 30% decrease in growth rate of CHO cells when the pCO_2 concentration was increased from 36 to 195 and 250mmHg, respectively. Gray *et al* (1996) demonstrated a 33% decrease in cell density at 148 mmHg in a large scale perfusion culture compared to a control at 36mmHg. In these studies osmolality was not controlled with increasing pCO_2 control, therefore, it is not possible to determine if the effect was due to an increase in pCO_2 or osmolality. The inhibition of the growth of hybridoma cells was observed by deZengotita *et al* (2002) when grown in T-flasks at elevated CO_2 conditions. A decrease in hybridoma cell growth was also observed in batch reactors by deZengotita *et al* (1998).

Zhu *et al* (2005) examined the effect of pCO_2 and osmolality separately by controlling osmolality under elevated pCO_2 levels. Elevating pCO_2 from 50 to 150 mmHg resulted in a 9% reduction in the specific cell growth. Osmolality had the greatest effect with a 60% decrease when increased from 316 to 450 $mOsm.Kg^{-1}$. The authors concluded that osmolality had a greater impact on cell growth than pCO_2.

The cause of the inhibitory effect of elevated pCO_2/osmolality on cell growth is not fully understood, with a number of studies contributing the decrease to osmolality, pCO_2 or a combination of both (Gray *et al,* 1996; deZengotita *et al*, 1998; Kimura and Miller, 1996). Possible theories on the effects of pCO_2/osmolality include the changes to the pH_i, which affects pH dependant processes within the cell, and the direct effect of elevated pCO_2/osmolality levels on the cell metabolism. The lack of

understanding arises from difficulties in analysing cell metabolism data (i.e. internal cell metabolism) and the interacting effects of other factors.

Cell metabolism – Lactate, ammonium and amino acids

The CHO cell metabolism is a highly linked multivariate system. Analysis of media amino acids gives an indication of the cell metabolism in terms of extracellular amino acids. However, there is no published data in the literature (to the author's knowledge) on the effect of pCO_2/osmolality on the amino acids metabolism. Instead, studies have been focused on lactate and ammonium. Although lactate and ammonium are key parameters in cell culture, the analysis alone does not give an indication in cell performance. This is due to the multivariate nature of cell culture, with numerous metabolism pathways possible for both metabolites.

Generally, in the literature, increasing pCO_2/osmolality has been reported to increase production of lactate and ammonium. Zhu *et al* (2005) reported an increase in lactate and ammonium production with elevated osmolality for a CHO cell line. An increase in lactate and ammonium production for a hybridoma cell line under increasing osmolality was also shown by Ozturk and Pallson (1990). Schmelzer and Miller (2002) reported an increase in lactate production rate of 50% for a CHO cell line when osmolality was increased from 230 to 480 mOsm.Kg^{-1} at a constant CO_2 of 40 mmHg.

As discussed previously, lactate and ammonium levels have been shown to impact on the cell culture processes. Therefore, if differences in pCO_2 level can affect lactate and ammonium metabolism, then, the process performance may also be impacted.

Internal cell pH - pH_i

A number of studies have shown that elevated pCO_2/osmolality affects the pH_i of the cell. Eisenkraetzer (2013) reported that elevated pCO_2 level led to the intensification of the oxidative metabolism (with the conversion of pyruvate to oxaloacetate for corporation into the TCA cycle) via an increase in pH_i. Both Eisenkraetzer (2013) and Silva *et al* (2013) reported that elevated pCO_2 levels triggered quicker progression of cells into G1/G0-phase of the cell cycle, reportedly a more productive cell stage when coupled with increased pH_i. Therefore, the effect of pCO_2 on pH_i may influence the mAb production in cell cultures. However, studies contradict the effect of pCO_2 on pH_i, demonstrating that cell line or process specifics, i.e. other interacting process parameters, have an effect on the process response (deZengotita *et al*, 2002; Eisenkraetzer *et al*, 2013; Silva *et al*, 2013). deZengotita *et al* (2002) reported a decrease of 0.2 of a hybridoma cell pH_i when pCO_2 was increased from 40 to 140 mmHg, with no further decrease at higher pCO_2 levels. However, Eisenkraetzer *et al* (2013) and Silva *et al* (2013) reported that elevated CO_2 led to an alkalisation (increase) in pH_i.

Garnier *et al* (1996) suggested that an optimisation of CO_2 transfer may be beneficial for increasing recombinant protein production in baculovirus infected Sf-9 (insect) cells. Under elevated CO_2 levels, a significant decrease in protein yield was observed, but an extended expression phase was noted in a 15% CO_2 controlled incubator compared to an air-controlled incubator. Introduction of CO_2 delayed the infection process by slowing the cellular metabolism of the cells. Therefore, an optimisation of the CO_2 control to extend the expression phase and increase productivity may be beneficial (Garnier *et al*, 1996).

1.3.3 Summary

Varied results have been reported on the effect of pCO_2/osmolality on productivity and product quality of mammalian cell cultures. Generally, elevated pCO_2 and osmolality have been reported to decrease product titre or had no effect due to a combination of increased productivity and reduced cell growth. The general understanding in the literature/industry is that elevated pCO_2 levels in industrial scale reactors are inhibitory to process performance. However, the majority of this knowledge has been gathered from a small number of studies conducted over ten years ago. During this time a number of significant advances have occurred in processing technologies, process monitoring and control, medium formulations (such as a move from serum to serum-free media) and cell lines. Additionally, the knowledge has come from analysing historical data, where high levels of pCO_2 has been present in poor performing cultures, however, this may be correlation as opposed to causation. More importantly, the limited studies on the effect of pCO_2 and osmolality have made use of unrepresentative vessels, such as micro-well plates and T-flasks, which lack the mixing and mass transfer, and monitoring and control capabilities of the large scale reactors. This results in the vessel being non-comparable and un-scalable due to differences in processing conditions and mixing (Bulnes-Abundis, 2013; Ozturk, 2014; Sandadi, 2013).

A number of studies did not consider the interacting effect of other un-optimised control parameters, such as the decoupling of pH and pCO_2 control. For example pH and pCO_2 cannot be decoupled in microtiter plates and T-flasks. As cell metabolic pathways are highly linked and multivariate in nature, a number of process conditions or unmonitored factors can affect the process as opposed to the factor being investigated, leading to misleading results. In industrial scale reactors pCO_2 builds up to higher levels than small scale vessels, mainly due to a combination comparatively poor mixing and gas stripping. Therefore, the build-up of pCO_2 is a consequence of differences in scale and process performance as opposed to the cause, as difference in process performance results in differences in pH control and the removal of pCO_2.

1.4 Research Objectives

Previous studies have demonstrated that CO_2 potentially affects cell line productivity and metabolism. The general understanding in industry/literature is that elevated pCO_2 levels lead to poor cell culture performance. However, elevated pCO_2 levels generally result from a combination of pCO_2 addition to control pH and poor gassing and mixing, as pCO_2 is not removed from the culture system from low mass transfer conditions. Therefore, elevated pCO_2 levels are an indicator of performance as opposed to the cause of the poor performance. Consequently, pH and pCO_2 control need to be decoupled in order to study the effect of pCO_2 on culture performance.

Currently, significant effort is directed towards the removal of CO_2 from large scale reactors in order to prevent the accumulation to high concentrations. However, the effect of pCO_2 level on a cell culture process and the optimal operating conditions (i.e. the amount of stripping required) are not well understood.

The CO_2 accumulation in a bioreactor is scale-dependant. Figure 1.2 indicates that the pCO_2 profile is not consistent across reactor scales. In laboratory scale vessels, CO_2 is easily removed, whereas, in industrial size reactors CO_2 accumulates to levels exceeding 100mmHg. In addition the time-based profiles for pCO_2 differs between scales, as shown in Figure 1.2. Therefore, if pCO_2 affects the critical quality attributes of a process, then its control is important for scalability and also for the validity of small scale studies.

For continuous processes, such as perfusion cultures, where a pseudo-steady state is achieved, a constant pCO_2 level can inherently occur. Therefore, for these cultures selection of an optimum pCO_2 level is required.

A review of published literature suggested that varying levels of pCO_2 affects the growth, productivity and product quality of a cell culture process. However, there is a lack of knowledge on the use of CO_2 sensors to control the level of pCO_2, independent of pH, in bioreactor systems. If the pCO_2 concentration affects the performance of cell culture processes, then the application at production scale would be beneficial and hence would require consideration across reactor scales.
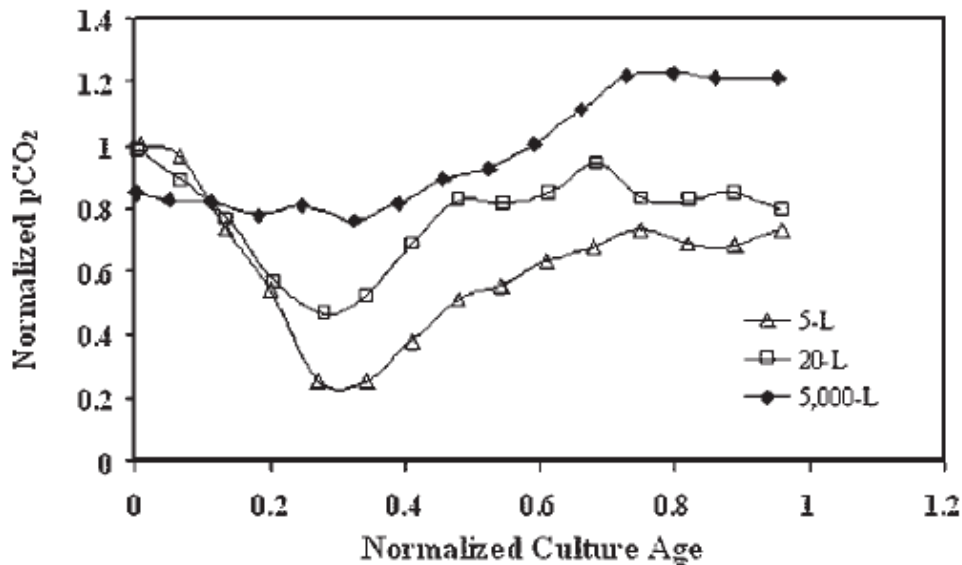
Figure 1.2 - Carbon Dioxide Profiles across Reactor Scales (5-5000L) during a Cell Culture Process (Xing et al, 2009) (Note: Carbon dioxide levels are normalised to the initial values at 5 L Scale)

The current research by the author, jointly funded by the ESPRC, Newcastle University and Pall Corporation, firstly investigates the effect of pCO_2 control on a CHO cell line and secondly undertakes a multivariate analysis of the data generated in order to gain further process knowledge.

The primary objective of the study is to examine the effect of pCO_2 on the performance, specifically the productivity and product quality, of a mAb producing CHO cell line and to determine if pCO_2 is a critical process parameter. The main responses examined are the productivity and product quality (the critical quality and performance attributes) of a cell culture process, as well as the effect of pCO_2 on the cell metabolism. It is also important to determine if pCO_2 control affects the reproducibility of a cell culture process, which is vital for production processes, due to administrative regulations.

The effect of pCO_2 control has been examined in bench-top STRs. The operation of bench-top scale STRs allows for the generation of a large amount of data on environmental, mechanical, chemical and biological parameters. The online/at-line data of variables which have been collected include; environmental process parameters (dO_2, pCO_2, pH and temperature), metabolites (glucose, glutamine, lactate, ammonium, etc.) and data on the cell count, size and viability. Offline data acquired includes amino acid concentrations and product quantity and quality aspects. The data collected has then been examined using principal component analysis to gain further knowledge and also to demonstrate the use of PCA for examining cell culture data.

The additional objective of the research is to develop a testing/operating procedure (for training and operation for other users at Pall Corporation) on the cell culture and bioreactor systems and at-line

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analytics. Additionally, cell culture runs will increase process knowledge on the cell line and STR operation for Pall Corporation to aid with scale-up and optimisation studies. The generation of historical data will allow for the development of further processes for bioreactor systems.

1.5 Work Structure

For simplicity and to avoid confusion to the reader the work structure has been laid out in a style that will enable the reader to follow the experimental rationale in a logical and simple manner. The chapters have been presented in the order:

CO_2 control level

CO_2 control and osmolality level

Fed-batch study

PCA investigation

2 Effect of Carbon Dioxide Control Level on a Mammalian Cell Culture Process

2.1 Introduction

The objective of the study described within the current chapter is to examine the effect of pCO_2 on the performance, specifically the productivity and product quality, of a mAb producing CHO cell line. Using bench-top scale STRs the effect of controlling pCO_2 at four levels has been compared to a baseline condition, which is a standard operating practice for CHO cell culture. For the baseline condition, pCO_2 is used to control the pH, i.e. increasing pCO_2 reduces pH and vice versa, with base addition when the use of pCO_2 alone is not sufficient. For the pCO_2 controlled conditions, pH and pCO_2 control are decoupled with the use of base and acid.

A number of parameters have been analysed to assess the performance, including cell metabolism (lactate, ammonium, amino acids), cell growth and mAb titre and quality. For the following chapters the results are presented followed by the discussion section.

Within the thesis the term 'exponential growth phase' has been used to describe the rapid growth phase post inoculation until the cells reach the maximum cell density and cell counts stabilise/decrease. Despite this phase not being truly exponential, it has historically been described as the exponential phase. Therefore, to adhere to the standard terminology, the following chapters refer to this phase as the exponential growth phase.

2.2 Materials and Methods

2.2.1 Cell Line and Medium

For the inoculation of the bioreactors, a CHO-S cell line expressing a humanised IgG1 monoclonal antibody that binds HER2 was maintained in the early exponential phase in 250 mL (100 mL working volume) Erlenmeyer shake flasks (Corning®, New York, USA). The CHO cells were maintained in CD FortiCHO™ medium (Life Technologies™, Grand Island, USA) supplemented with UltraGlutamine Supplement (Lonza, Verviers, Belgium or equivalent), 100X HT Supplement (Life Technologies) and Puromycin Dihydrochloride (Life Technologies). The shake flasks were incubated at $37^{o}C$ in a humidified atmosphere containing 8% CO_2, using controlled incubators (Triple Red, Buckinghamshire, UK). Agitation was provided by an orbital shaker at 135 RPM (Stuart Equipment,

Staffordshire, UK). Cells from the shake flasks at the mid-exponential growth phase were taken directly into 2.5 L bioreactors for cell culture runs.

2.2.2 Bioreactor Cell Culture Runs

For the context of this study, a 'run' is defined as an individual STR cell culture and a 'batch' is a group of STR runs carried out from the same inoculum, i.e. inoculated from the same shake flask source. For each batch a baseline condition (i.e. no CO_2 control) was carried out, in order to normalise response data and hence to compare data across batches, i.e. in order to account for batch-to-batch variation.

2.5 L CelliGen® 310 bioreactors (Eppendorf, Hamburg, Germany) were inoculated to a working volume of 1.4 L, at a seeding density of $5x10^5$ cells.mL^{-1} and a viability greater than 95%. The STR cultures were carried out using a CD FortiCHO medium (Life Technologies), supplemented to 4mM UltraGlutamine Supplement (Lonza) and 10 mL.L^{-1} HT supplement (Life Technologies, Grand Island, USA) and 15 g.L^{-1} glucose (Sigma-Aldrich®, Missouri, USA). Glucose levels were increased to 10 ± 1.0 g.L^{-1}, with a 45% glucose solution, once the value fell below 4 g.L^{-1}. Cell cultures were harvested at 50 ± 10% viability, which occurred at approximately 312 hours.

The bioreactors were fitted with an elephant ear impeller and ring sparger for mixing and aeration. Process set points were maintained by CelliGen 310 control towers with Reactor Process Control software (Eppendorf).

The pH was measured using a 405 DPAS-SC-K8S gel-filled electrode (Mettler-Toledo, Massachusetts, USA), dissolved oxygen was monitored with an InPro 6830 polarographic electrode (Mettler-Toledo) and pCO_2 was measured via an InPro 5000 Severinghaus principle probe (Mettler-Toledo).

Each STR was configured with three mass controllers (with a gas flow range of 0.02 to 1.0 standard litre per minute, SLPM) to supply compressed air, oxygen and carbon dioxide through a 0.2 µm bacterial air vent (Pall® Corporation, New York, USA). The air flow was controlled to a total flow of 0.1 SPLM for baseline runs, with carbon dioxide flow cascaded to pH control and oxygen to DO control. Air flow was set to 0.1 SPLM for CO_2 controlled runs, with CO_2 and O_2 gas flows cascaded to pCO_2 and DO sensors, respectively.

The controller set-points were set to: pH 7.20 (±0.03), DO 40% (±10), temperature 37°C (±0.1) and agitation 200 rpm (±5). The pH was adjusted using 1M NaOH and HCl for CO_2 controlled runs, to decouple pH control from CO_2 and 1M Na_2CO_3 and CO_2 gas in the baseline runs. The effects of CO_2

controlled runs were compared against baseline runs (no CO_2 control), which represent industrial standard STR cell cultures.

The fixed parameters for all STR runs are summarised in Table 2.1, shown with the acceptance criteria, i.e. the study controls.

Parameter	Acceptance Criteria	Units
Seed harvest viability	>95	%
Seed harvest density	3.00 ± 1.00	10^6 cells.mL^{-1}
STR seeding density	0.5 ± 0.10	10^6 cells.mL^{-1}
STR total fill volume	1400	mL
STR agitation	200 ± 5	Rpm
STR temperature	37.0 ± 1.00	^{o}C
STR dissolved oxygen	40 ± 10	%
STR pH	7.2 ± 0.03	Units
STR pH control	1 M HCl & 1 M NaOH 1 M Na$_2$CO$_3$ & CO$_2$	-
STR Harvest viability	60 ± 10	%

Table 2.1 - Bioreactor Acceptance Criteria for Controlled Parameters

2.2.3 pCO$_2$ Control Levels

The pCO$_2$ control levels used in the study are summarised in Table 2.2.

Factor	Levels	Description
pCO$_2$ (mmHg)	5	Baseline, 20, 60, 100, 140 (±10)

Table 2.2 - STR pCO$_2$ Control Conditions

The maximum pCO$_2$ control level was selected as 140mmHg, as this was the highest ideal working range for the in-line (pCO$_2$ sensor) and offline (Nova Bioprofile Flex) gas/metabolite analytics. The STR control conditions for each batch are given in Table 2.3.

Batch	STR Runs in Batch	pCO$_2$ Level (mmHg)
1	4	Baseline (x2), 60,100
2	5	Baseline (x2), 20, 60, 100
3	6	Baseline , 20, 60, 100, 140 (x2)
4	5	Baseline , 20, 100, 140 (x2)

Table 2.3 - STR Conditions Examined in Each Batch. Note: x 2 indicates a duplicate run with in the batch

The original study plan called for single points for each pCO$_2$ control level and duplicate baseline runs within each batch, carried out across three batches. However, due to STR equipment failures within batches (agitator motor and temperature probe failures) the STR run plans had to be modified to those shown in Table 2.3.

2.2.4 At-line Analytics

Each individual STR was connected to a BioProfile® Flex Analyser (Nova® Biomedical, Waltham, Massachusetts) with an integrated autosampler for at-line sample acquisition. A reactor valve module (RVM), one per STR, took a 10mL sample every 24 hours, for analysis by a gas/metabolite analyser, providing the parameters summarised in Table 2.4. The Nova Flex was used to analyse key parameters in the cell cultures to indicate and compare performance, such as the cell growth and metabolism.

The Nova flex auto-calibrates every two hours using standards supplied by Nova Biomedical. In addition, daily quality control (QC) standards were run on the Nova Flex (supplied by Nova Biomedical) to check the calibration (i.e. low, mid and high value) for each parameter. The QC ranges are provided in Appendix 8.1. In-line sensors (pH and CO_2) were checked for drift with offline syringe samples via the Bioprofile Flex and were adjusted, if required.

Module	Parameter	Measurement Type
Nutrient/Metabolite	Glucose	Biosensor
Nutrient/Metabolite	Lactate	Biosensor
Nutrient/Metabolite	Glutamine	Biosensor
Nutrient/Metabolite	Glutamate	Biosensor
Gas/Electrolyte	Ammonium	Direct Ion-Selective Electrode
Gas/Electrolyte	pH	Direct Ion-Selective Electrode
Gas/Electrolyte	pCO_2	Direct Ion-Selective Electrode
Gas/Electrolyte	pO_2	Clarke Electrode
Gas/Electrolyte	Sodium	Direct Ion-Selective Electrode
Gas/Electrolyte	Potassium	Direct Ion-Selective Electrode
Gas/Electrolyte	Calcium	Direct Ion-Selective Electrode
Osmolality	Osmolality	Freezing Point
Cell Density/Viability	Viable cell count	Digital Imaging (Trypan blue exclusion)
Cell Density/Viability	Total cell count	Digital Imaging (Trypan blue exclusion)
Cell Density/Viability	Cell diameter	Digital Imaging

Table 2.4 - Nova Bioprofile Flex At-line Parameters and Measurement Type

2.2.5 Offline Analytics

All offline analytics (Table 2.5) were carried out under the supervision of or by the Applications R&D Analytics teams at Pall Corporation (Portsmouth). Product quantity was determined by protein-A affinity chromatography on a Prominence HPLC (Shimadzu, Kyoto, Japan) using PDA (UV) detection. Charge variants were analysed using weak cation exchange chromatography on a Classic Acquity UPLC® (Waters, Milford, USA). Amino acid concentration was determined by reverse phase AccQ-tag™ derivatisation UPLC on a Classic Acquity UPLC (Waters).

Offline Analytic	Assay Type	Measurement Instrument
Amino Acid Concentration	Reverse phase AccQ-tag™ derivatisation	UPLC
mAb Charge Variants	Cation exchange	UPLC
Total mAb Concentration	Protein-A affinity	HPLC

Table 2.5 - Offline Analytics and Assay Type

2.3 Results

Results from the pCO_2 controlled runs within a batch were normalised over the baseline condition for that batch. For example, for the harvest mAb titre, the value at each pCO_2 control level was divided by the baseline value. The mean and standard errors were calculated from the normalised values.

2.3.1 Antibody Production

mAb Titre
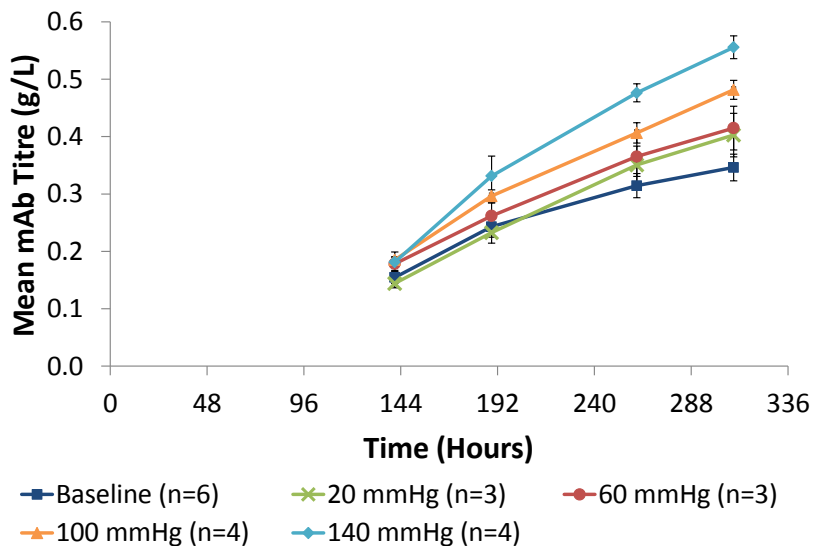
Figure 2.1 - Mean mAb Titre for all pCO_2 Control Levels (Note: Shown with S.E. of the mean and data has not been normalised against the baseline conditions)

Samples for mAb titre were taken post 144 hours, when the cells reached the stationary phase of growth as mAb levels were below the detection limit of the analytical equipment prior to this. For all conditions mAb titre increased with time, as shown in the time series plot of mAb titre in Figure 2.1. Little difference was seen in the mAb production at 144 hours, approximately the start of the stationary phase of growth, after which the production levels for the various conditions deviated from each other. For the 140, 100 and 60 mmHg conditions, the antibody titre exceeded the baseline conditions throughout the process, whilst for the 20mmHg condition it only exceeded the

baseline value post 192 hours. The lowest mAb titre was obtained in the baseline conditions and the greatest for the 140 mmHg conditions. For the baseline condition, the mAb titre increased from 0.154 to 0.346 g.L^{-1} and from 0.181 to 0.556 g.L^{-1} for the 140 mmHg condition.

Increasing pCO_2 control from 20 to 140 mmHg resulted in an increase in harvest mAb titre compared to the baseline conditions, as shown in Figure 2.2. At an elevated pCO_2 control level (140 mmHg), a 40% increase was observed, with a 10, 20 and 35% increase at 20, 60 and 100 mmHg over the baseline, respectively.

The pCO_2 condition at 100 mmHg has an error bar which is approximately 120% greater than that of the other conditions. This is likely due to the fact that at 140 mmHg a change to the cell metabolism occurs (resulting in an increase in mAb titre as discussed later in this chapter, Sections 2.3.3 and 2.3.4). It is thus hypothesised that the 100 mmHg condition is at the "borderline" in terms of mAb production, between the 140 mmHg and the lower pCO_2 control conditions, resulting in an increase in variability.
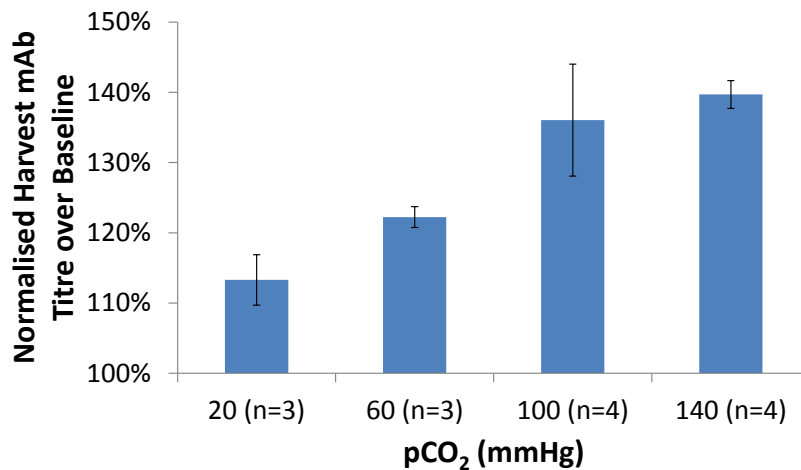
Figure 2.2 - Harvest mAb Titre Normalised over the Baseline Condition within Each Batch (Note: Shown with S.E. of the mean calculated using the normalised values)

Cell Specific Productivity

The cell specific mAb production rate was calculated from the start of the stationary phase (144 hours) to the harvest point (312 hours):

$$\frac{dmAb}{dt} = q_{mAb}X_v \qquad (2)$$

$$mAb = q_{mAb} \int_{t1}^{t2} X_v \, dt$$

Where q_{mAb} is a constant

The cell specific antibody production rate (q_{mAb}) is determined by plotting the antibody concentration (mAb) against the integral viable cell concentration ($\int X_v dt$), where X_v is the viable cell concentration. For all conditions, a straight line was obtained, indicating constant productivity and demonstrating that equation (2) is valid.
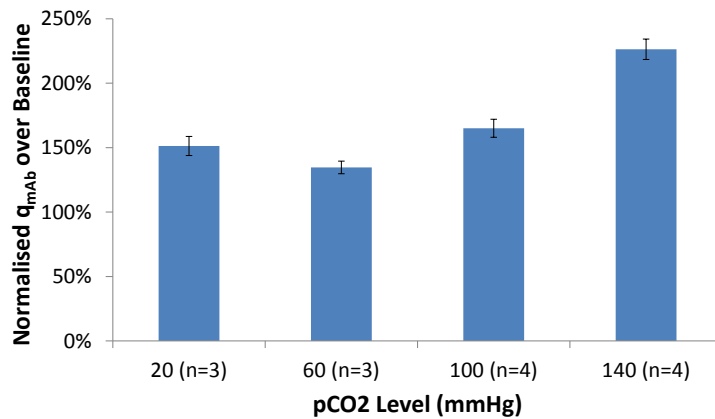
Figure 2.3 - Cell Specific Antibody Production Rate Normalised to Baseline Cell Specific Productivity (Note: Shown with S.E. of the mean calculated using the normalised values)

Figure 2.3 illustrates the effect of pCO_2 control on the cell specific production rate, with the values normalised against the baseline for each batch. The pCO_2 control resulted in an increase in the cell specific productivity, with a 35-65% increase for conditions 20 to 100 mmHg and 130% increase at 140 mmHg. Due to the sensitivity of cell counts and the method for calculating q_{mAb}, the differences between q_{mAb} values for the 20 to 100 mmHg conditions are not considered significant. This is due to the fact that the calculation makes use of measured values for cell density, which can vary on the instrument up to 10% at levels of 16 million cells.mL^{-1}, which can affect the calculation. Also, the calculation of q_{mAb} makes use of a line fit, which can have an inherent error associated with it (i.e. model fit error). Therefore, the difference between the 20 and 60 mmHg conditions is not considered significantly different from each other, whilst the increase at 140 mmHg is significant compared with the other conditions.

2.3.2 Product Quality

Charge variant analysis provides the relative composition of acidic, basic and target (the desired form) mAb of the mAb product; where the sum of the target, acidic and basic mAb is 100%. The target mAb is the desired mAb, with the basic and acidic variants being the undesired form.

Charge variant analysis can only be performed from 144 hours onwards as the mAb concentration is below the detectable limit required for the charge variant assay before this time.

Charge Variants

Controlling pCO_2 from 20 to 100 mmHg resulted in a lower target mAb composition compared to the baseline, with a difference of up to 10%, as shown in Figure 2.4 (a). Operating at 140 mmHg resulted in an equivalent target mAb composition compared to the baseline.

(a)

(b)

(c)
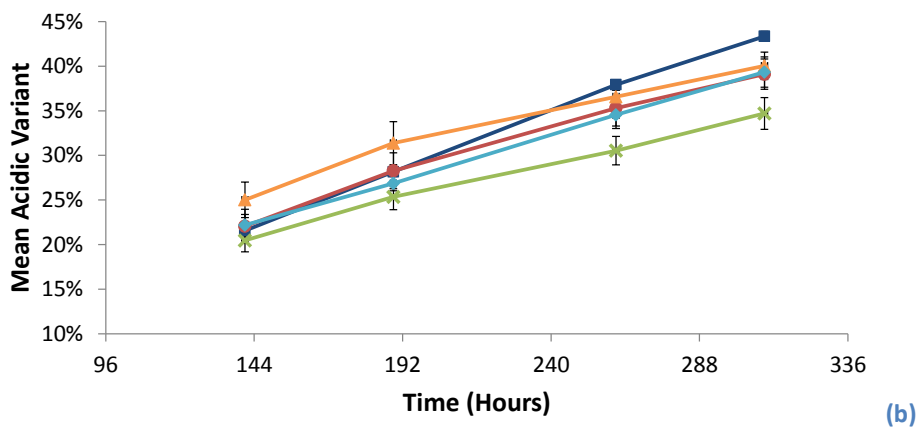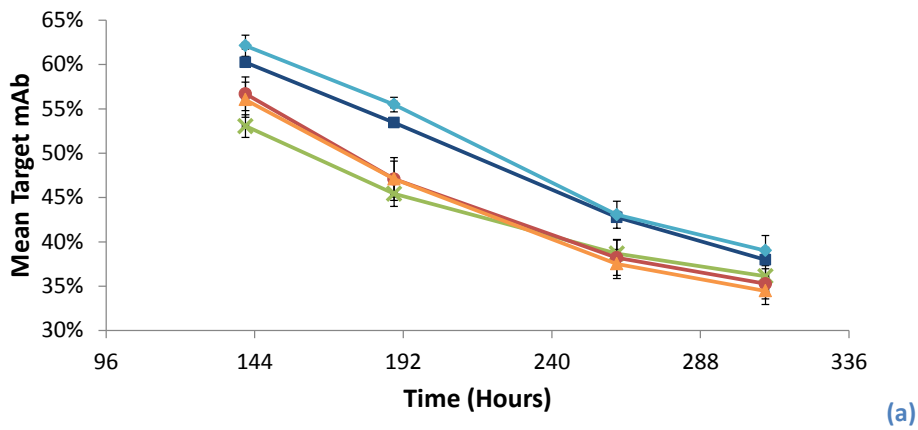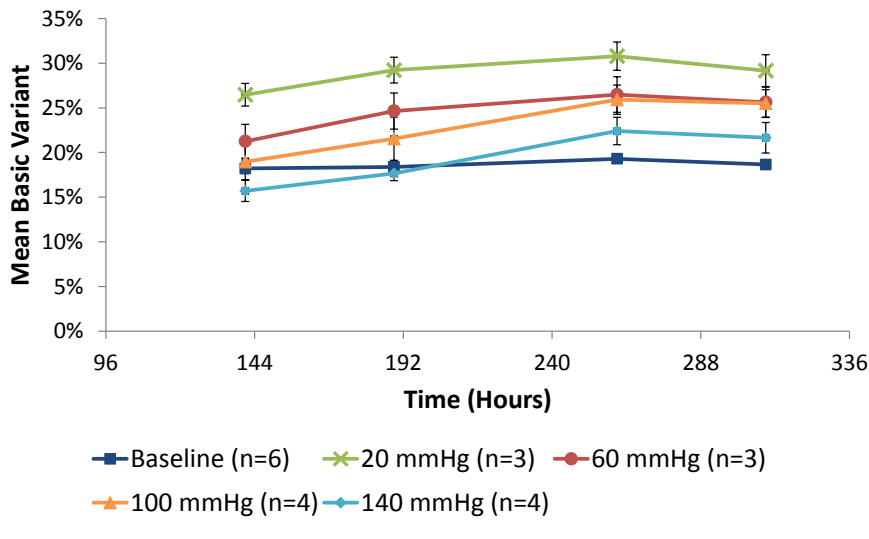
Figure 2.4 - Time Profile of mean (a) Target mAb (b) Acidic Variants and (c) Basic Variants Composition
(Note: Shown with S.E. of the mean)

The target mAb profile is correlated to the cell viability, i.e. as time progresses and cell viability decreases, the target mAb composition decreases (Figure 2.4 (a) and Figure 2.9 (a)). This is hypothesised to be due to a combination of the degradation of the target mAb with time and the release of toxic metabolites with decreasing cell viability.

The maximum composition of target mAb measured for all conditions was present at the start of the stationary phase, approximately 144 hours. The baseline and 140 mmHg conditions had the greatest amounts with 60% and 63%, respectively. The 20 mmHg condition had the lowest target mAb composition at the start of stationary phase, with 52% and the 60 and 100 mmHg conditions have a similar target mAbs of 56%.

Figure 2.5, the gradient of the linear fit for each mAb variant, indicates that there was less than 6% difference between the decline in target mAb for the baseline, 60, 100 and 140 mmHg conditions. For the 20 mmHg, there was a 16% lower decrease in target mAb, compared to the baseline.
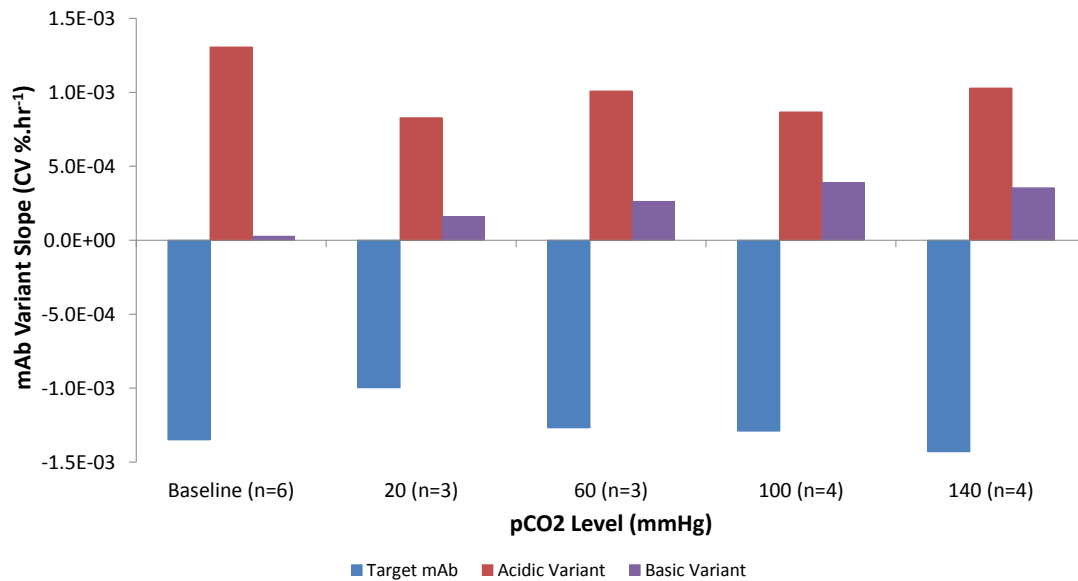
Figure 2.5 - Gradient of the Linear Fit of Each mAb Variant during the Cell Culture

The formation of acidic variants was the major cause of the decrease in target mAb during the cell culture. The linear nature of the degradation suggests that it is time dependant, and hence due to the culture environment, i.e. toxic metabolites released by dying cells. Figure 2.4 (b) shows a less than 5% difference between the initial acidic variants for all conditions (144 hours), with the lowest at 20 mmHg and greatest at 100 mmHg, being 20.5% and 25%, respectively. The increase in acidic variants during the course of the culture is greatest for the baseline at 22% compared to 14-17% for the 20 to 140 mmHg conditions.

Figure 2.5 illustrates that the increase in acidic variants was the main cause of the decrease in the target mAb composition. The greatest decline in acidic variants was seen for the baseline condition with a 37, 23, 34 and 22% lower decline for the 20, 60, 100 and 140 mmHg conditions, respectively. When taking into account the assay error for the acidic variants and the small values of the gradients, the differences between the 20 to 140 mmHg are not considered to be considerably different.

Reducing pCO_2 control results in an increase in basic mAb composition compared to the baseline condition. At the end of the culture there is a 10% increase over the baseline at 20 mmHg, 7% at 60 and 100 mmHg and 3% at 140 mmHg, shown in Figure 2.4 (c). During the course of the culture there was little change in the basic variants for each condition, with an increase from 0.4% to 6% from the baseline to 140 mmHg condition. This is reflected in Figure 2.5, which indicates that there is little change in the basic variants compared to the change in acidic variants. Figure 2.5 also indicates the increasing rate of formation of the basic variants with increased pCO_2 level.

2.3.3 Cell Growth

Viable Cell Growth Profile and Maximum Cell Titre

Control of pCO_2 from 20 to 100 mmHg had limited impact on cell growth (growth rate and maximum cell titre) compared to the baseline condition as demonstrated in Figure 2.6 and Figure 2.7. The pCO_2 control at 140 mmHg resulted in suppressed cell growth, indicated by a lower maximum cell titre and specific growth rate.
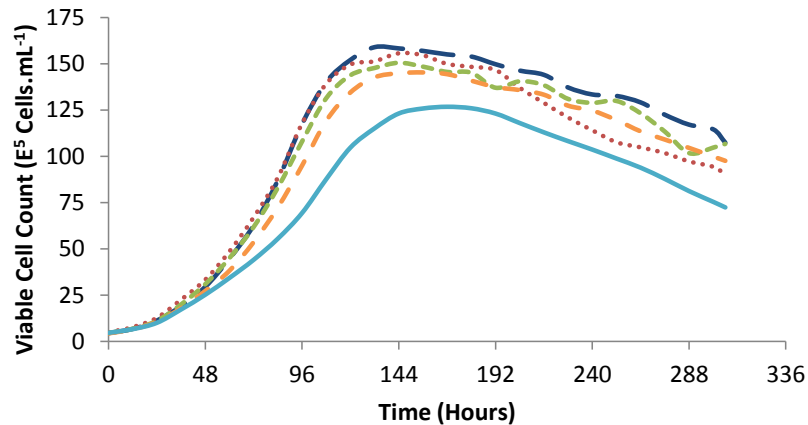
Figure 2.6 - Time Profile of Mean Viable Cell Count for pCO₂ Control Levels (Note: Data points have been interpolated to new equal time points by linear interpolation to determine mean values)

Figure 2.7 indicates a decrease in maximum cell titre with increasing pCO_2, with a 2 to 10% drop from 20 to 100 mmHg and a 20% decrease at 140 mmHg. Elevated pCO_2 control results in increased osmolality, with both parameters having previously been shown to impact cell growth (Gray *et al*, 1996; deZengotita *et al*, 1998; Kimura and Miller, 1996; Zhu *et al*, 2005). The associated osmolality increase is due to the addition of base during the course of the culture (primarily at the start of the culture, prior to inoculation), leading to an accumulation of Na^+. Increased base addition is required with increasing pCO_2 control level to offset the effect of pCO_2 on the pH.
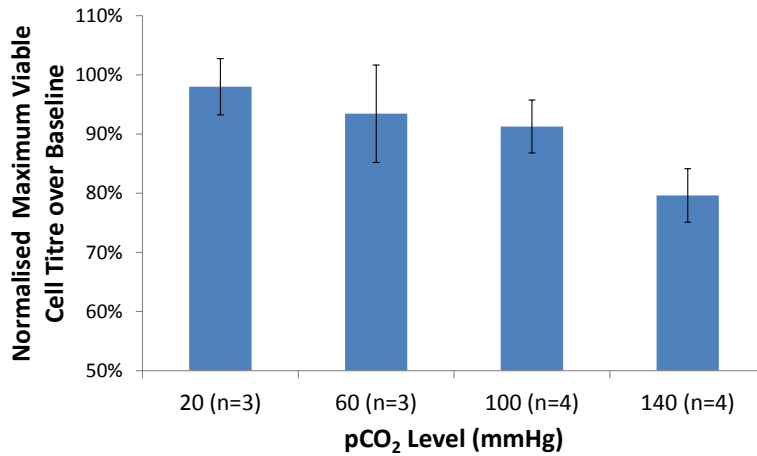
Figure 2.7 - Mean Maximum Viable Cell Titre for pCO$_2$ Control Levels Normalised to the Baseline within Each Batch (Note: Shown with S.E. of the mean)

Error bars of up to 10-15%, for Figure 2.7, indicates the variability of the cell count assay, which is inherent in the analytics; the automated trypan blue dye exclusion method for cell counting via camera images. This error is exemplified at large cells counts, as above 1×10^6 cells.mL^{-1} a dilution (1:2) of the sample is required for counting.

Specific Growth Rate

The effect of the pCO$_2$ control level on the specific growth rate is shown in Figure 2.8. The specific growth rate (μ) is calculated from the gradient of the natural log of viable cells (X_v) against time during the exponential growth phase:

$$\frac{dX_v}{dt} = \mu X_v \qquad \textbf{(3)}$$

At a pCO$_2$ control level of 20 to 100 mmHg, there is minimal effect (less than 5%) on the specific growth rate but a 15% reduction is observed at 140 mmHg, compared to the baseline.
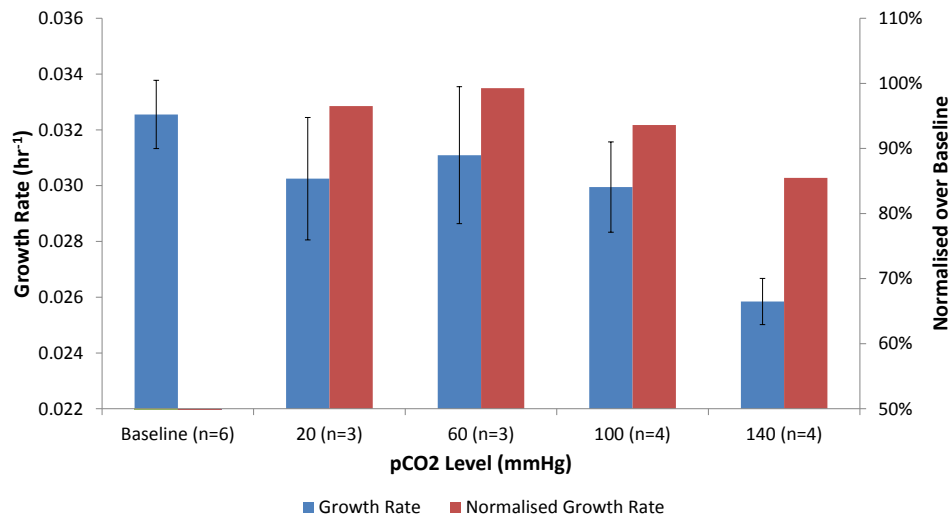
Figure 2.8 - Effect of pCO$_2$ Control Level on Specific Growth Rate of Viable Cells and the Normalised Value against the Baseline (Note: Shown with the S.E. and the specific growth rate is calculated from the gradient of the natural log of viable cells against time during the exponential growth phase)

2.3.4 Cell Metabolism

Control of pCO$_2$ resulted in a change in the cell metabolism, demonstrated by a change in the ammonium, lactate and amino acid profiles and concentrations.

Lactate and Ammonium – Key Process Indicators

Figure 2.9 (b), the ammonium time series plot for all conditions, indicates an increase in ammonium concentration with culture time. For the baseline and 20 to 100 mmHg conditions, a re-metabolism of ammonium occurred from approximately 72 to 144 hours, which is a known to occur in CHO-S clones. However, for the 140 mmHg condition at the same time point (mid exponential phase), the production rate of ammonium is slowed, but ammonium is not re-metabolised.

For all conditions a similar lactate profile to the baseline condition was observed, with production from the start of the culture to the late exponential phase (approximately 96 hours), at which point a shift to consumption occurred, shown in Figure 2.9 (c). Controlling pCO$_2$ at 20 and 60 mmHg resulted in a similar lactate profile to the baseline condition. However, operating at 100 and 140 mmHg resulted in a delay in the shift of lactate metabolism, which was equivalent to the delay in the shift from exponential to stationary growth (shown in Figure 2.9 (a)).
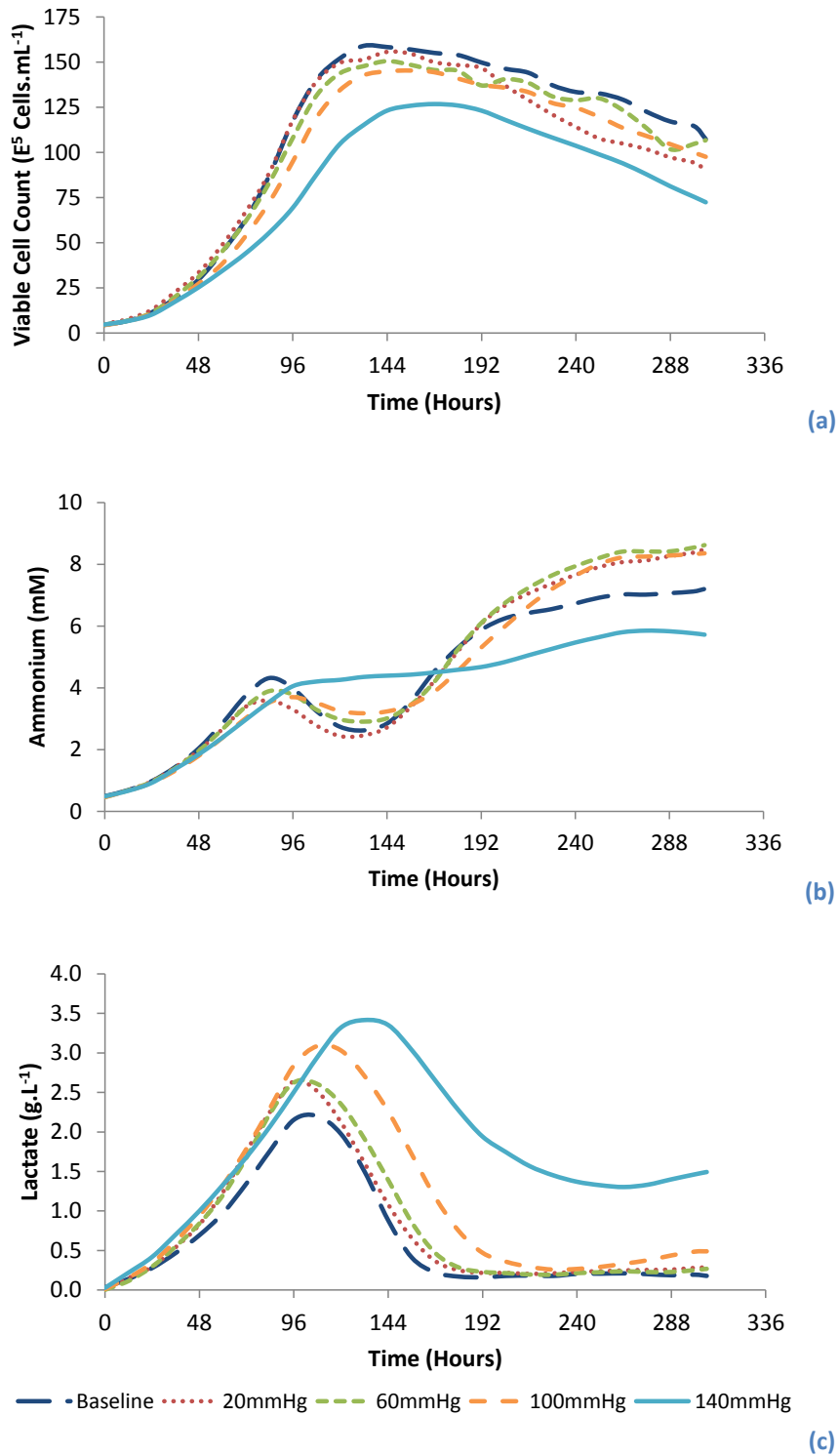
Figure 2.9 - Time Profile of Mean (a) Viable Cell Count (b) Ammonium (c) Lactate for pCO₂ Control Levels

(Note: Data points have been interpolated to new equal time points by linear interpolation to determine mean values)

A 10 to 20% increase in harvest ammonium concentration compared to the baseline condition was observed when increasing pCO_2 from 20 to 100 mmHg, as shown in Figure 2.10. At 140 mmHg, the ammonium titre was significantly affected, with a 30% reduction in the harvest ammonium concentration compared to the baseline.
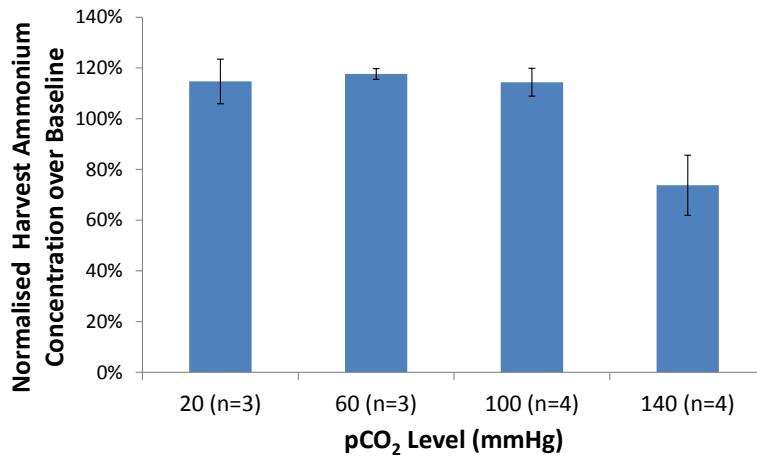
Figure 2.10 - Mean Harvest Ammonium Concentration for pCO_2 Control Levels Normalised to the Baseline for Each Batch (Note: shown with S.E. of the mean)

Increasing pCO_2 control resulted in increased maximum lactate levels and led to a delay in the re-metabolism of lactate. Compared to the baseline condition, a 20% increase at 20 and 60 mmHg, 40% at 100 mmHg and 50% at 140 mmHg, was observed in maximum lactate concentrations, shown in Figure 2.11.
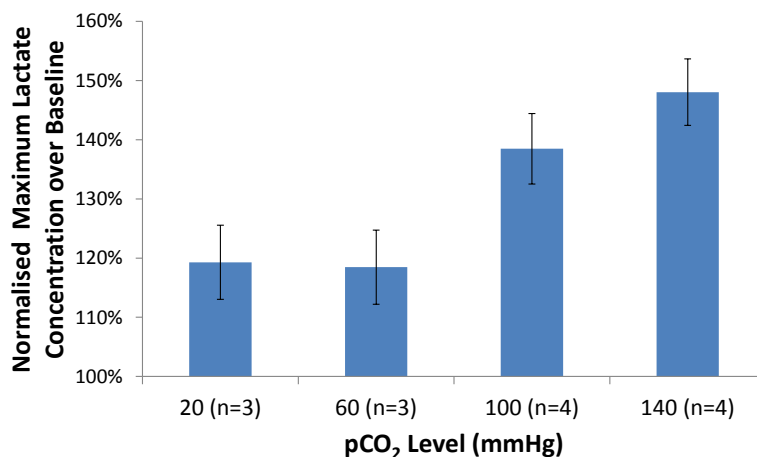
Figure 2.11 - Mean Maximum Lactate Concentration for pCO_2 Control Levels Normalised to the Baseline within Each Batch (Note: Shown with S.E. of the mean)

Key Amino Acids

Amino acids that showed a significant change due to pCO_2 control or which are believed to have a significant impact on CHO cell culture, from literature and observations, are presented in the following section.

L-alanine-L-glutamine (Glx) and asparagine (Asn) are key amino acids for cell growth (Dean and Reddy, 2013; Duarte *et al*, 2014; Li *et al*, 2012). Glx and Asn metabolism are closely related, as shown in Figure 2.12, with the consumption from the start of the culture to depletion at the mid-point of the exponential phase. Little difference is seen in the metabolism of Glx and Asn in the baseline and 20 to 100 mmHg conditions, however, a delay in depletion was observed for the 140 mmHg condition. The delay in depletion of Glx and Asn coincides with the delay in the switch of the lactate metabolism from production to consumption (Figure 2.9 (c)). It is possible that the reduced consumption rate of Glx/Asn is due to the reduced growth rate and number of cells or due to the effect of elevated pCO_2 control on the cell metabolism.
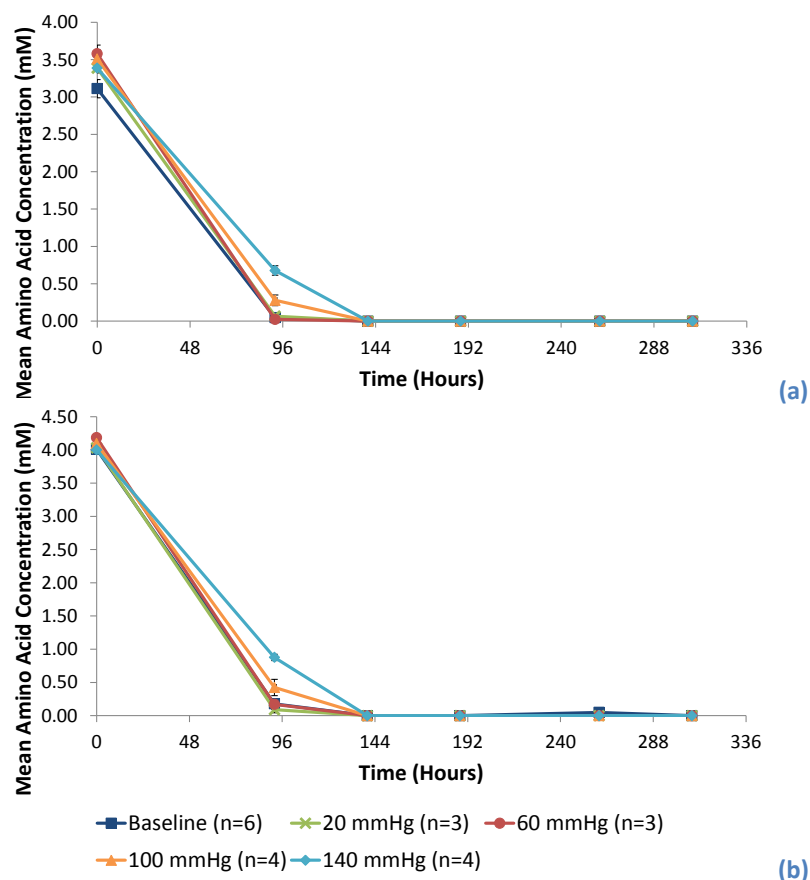
Figure 2.12 - L-alanine-L-glutamine (a) and Asparagine (b) Concentration Time Profiles for pCO_2 Control Conditions (Note: Shown with S.E. of the mean)

Control of pCO_2 significantly affected the metabolism of glycine, alanine and glutamate, as illustrated in Figure 2.13. The final mean glycine concentration for the baseline condition was the highest at 2.6 mM, with the lowest being recorded at 1.1 mM at 140 mmHg. For the baseline condition, glycine is produced until the start of the stationary phase, after which the rate of (net) production is significantly slowed, which occurs in both the 20 and 60 mmHg conditions. Operation at 100 and 140 mmHg resulted in the consumption of glycine, from the start of the stationary phase.

The alanine and lactate profiles (Figure 2.13 (b) and Figure 2.9 (c)) are closely related, due to the metabolism of Glx and pyruvate, which is derived from glucose and Glx (among other amino acids). An initial production phase for alanine and lactate is seen from the start of the culture until the mid to late-exponential phase, after which alanine is consumed at a similar rate to lactate. The most significant difference between the pCO_2 control levels is the opposing trend in maximum alanine and lactate levels, with the highest alanine and lowest lactate levels occurring in the baseline condition. At 140 mmHg, the lowest alanine and greatest lactate levels were observed, as well as the lowest alanine consumption rate during the stationary phase. The reduced alanine consumption rate is linked with a decrease in ammonium titres (Figure 2.13 (b) and Figure 2.9 (b)), which agrees with the hypothesis of Li *et al* (2012) that alanine consumption is the primary source of ammonium accumulation once lactate has been depleted. The build-up of alanine is due to a combination of the breakdown of Glx to alanine and glutamine, the metabolism of glutamine and the production of alanine from pyruvate.

The glutamate profile for pCO_2 control conditions from 20 to 140 mmHg show little difference with a linear increase from approximately 2.4 mM to 4.9 mM. In the baseline condition, glutamate is produced at a higher rate, from 2.4 mM to 6.4 mM.
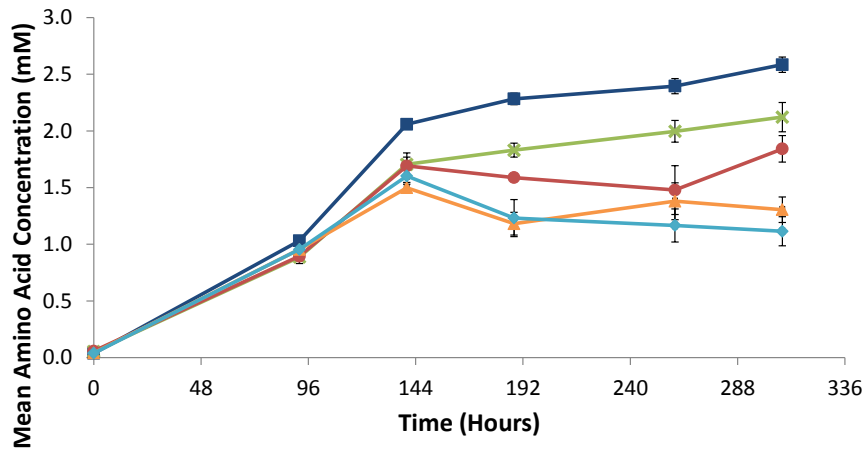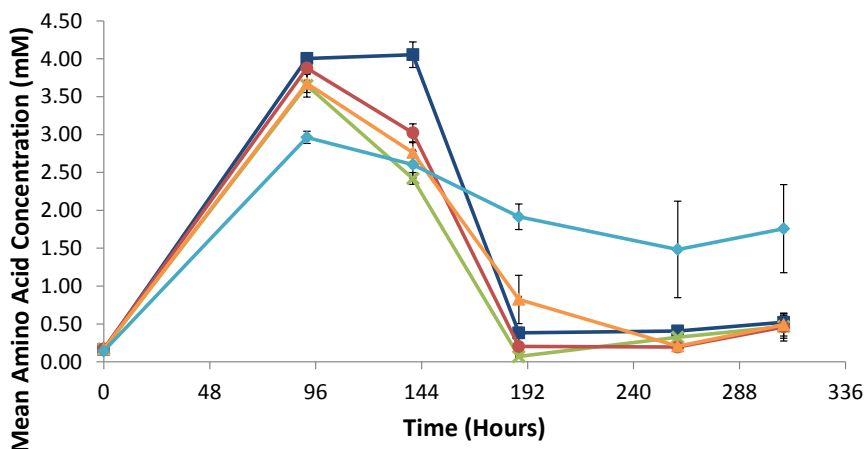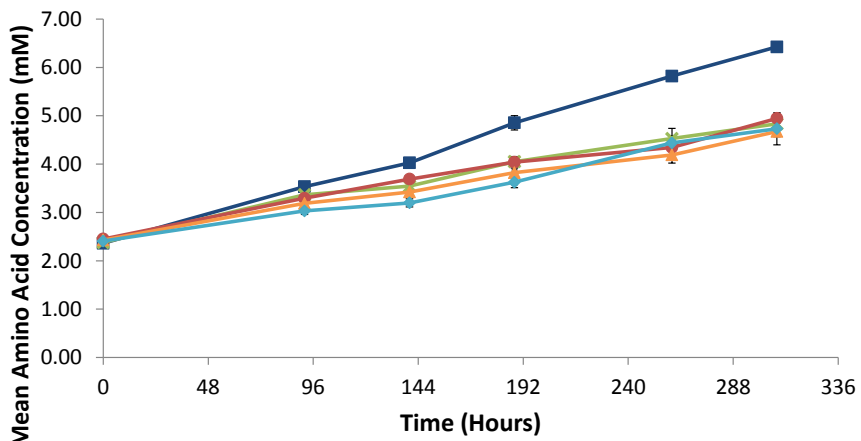
Figure 2.13 - Glycine (a), Alanine (b) and Glutamate (c) Concentration Time Profiles for pCO₂ Control Conditions (Note: Shown with S.E. of the mean)

2.4 Discussion

2.4.1 Antibody Production
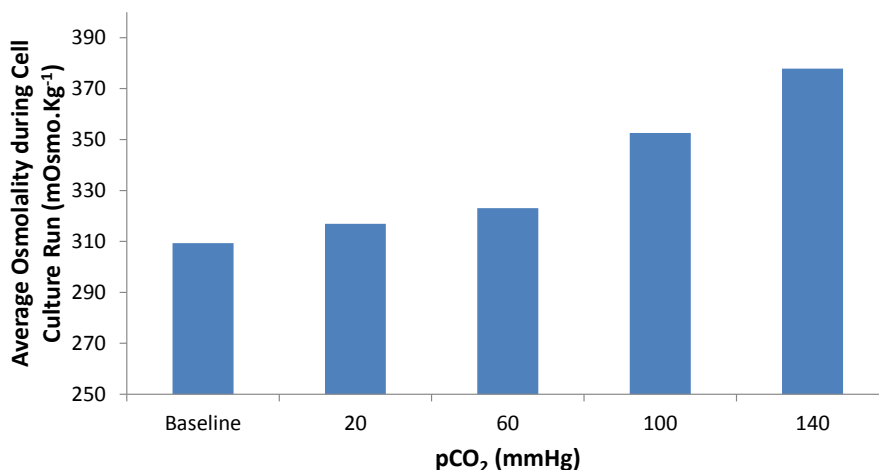
Figure 2.14 - Average Osmolality during Cell Culture Runs at Each Condition (Note: Error bars not shown as average value of time dependant variable)

Increasing pCO_2 control resulted in an increase in osmolality (Figure 2.14). This was mainly due to NaOH additions to control pH as well as differences in metabolism by-products (i.e. lactate and ammonia), leading to a possible interacting effect of pCO_2 and osmolality. Therefore, the term "pCO_2 control" encapsulates both the operation at controlled pCO_2, with the associated change in osmolality.

Increasing the pCO_2 control set-point, decoupled from pH control, resulted in an increase in mAb harvest titre and cell specific productivity compared to a traditional cell culture process (Figure 2.2). Previous studies have shown contrasting results in terms of the effects of elevated pCO_2 and osmolality on mAb production, however published studies are limited in number. A number of studies have stated that elevated osmolality and pCO_2 increase productivity (Brady, 2009; deZengotita *et al,* 1998; Eisenkraetzer *et al*, 2013; Ozturk and Palsson, 1990; Silva *et al*, 2013), whilst other studies have reported that elevated pCO_2 and osmolality have a negative impact on cell culture processes (Drapeau *et al*, 1990; Gray *et al,* 1996; Kimura and Miller, 1996).

The general understanding in the literature/industry is that elevated pCO_2 in bioreactors is inhibitory to process performance, contrary to the results found in this study. However, the current assertions have been based on limited studies in which unrepresentative vessels, such as micro-well plates and T-flasks, were used for cell culture runs, which lack comparability to STRs (Bulnes-Abundis, 2013;

Ozturk, 2014; Sandadi, 2013). T-flasks and well plates, Figure 2.15, achieve aeration via diffusion through the liquid surface, in environmentally controlled incubators.
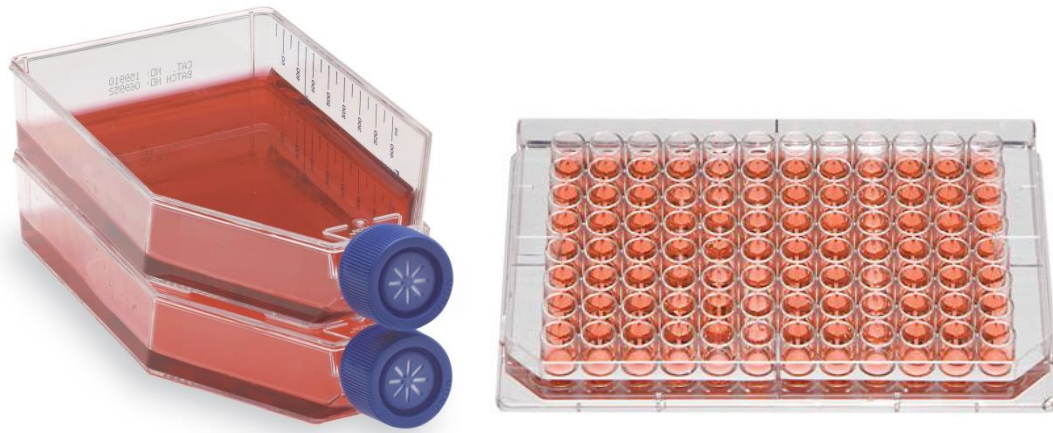
Figure 2.15 - Example T-Flask (T-Flask, n.d.) and Well Plate (Well Plate, n.d.) Culture Systems

As oxygen is sparingly soluble, the culture aeration in static systems is significantly limited (Ozturk, 2014). T-flask operation involves no mixing and micro-well plates achieve mixing through agitation of the entire plate. Therefore, the mixing and aeration of these systems are significantly different from STRs, which make use of direct gas sparging to the media and mixing via impellers. Equations (4) to (6) describe how the agitation rate and air flow directly affect the oxygenation of a STR bioreactor system.

$$OTR = k_L a \, (C^* - C) \qquad \text{(4)}$$

$$k_L a = A \left(\frac{P}{V}\right)^\alpha (\omega_{sg})^\beta \qquad \text{(5)}$$

$$\frac{P}{V} = P_0 N^5 D_i^3 \rho \qquad \text{(6)}$$

For sufficient aeration of a cell culture system the oxygen transfer rate (OTR) must be equal to or greater than the oxygen uptake rate (OUR), which is dictated by the cell metabolism and cell concentration. The OTR in the STRs is determined by the mass transfer coefficient ($k_L a$, hr^{-1}) and the concentration gradient (C* - C), where C is the oxygen concentration in the liquid phase and C* the concentration of oxygen in the liquid at saturation. Equations (5) and (6) demonstrate that the impeller speed (N, s^{-1}) and superficial gas velocity (Wsg, the volumetric air flow rate divided by the cross sectional area, m.s^{-1}) affect the magnitude of the $k_L a$ and, in turn, the oxygen transfer rate, equation (4). There are a large number of correlations developed for estimating $k_L a$ from agitated and sparger systems, but the most commonly used is Van't Riets, equation (5) (Nienow, 2015). In

equation (6), P_0 is the ungassed power number (dependent on the impeller type, D_i the impeller diameter and ρ the fluid density). A, α and β are constants in equation (5) relating to the STR system, i.e. impeller type, reactor geometry and also the liquid composition.

Aeration in static and micro-scale cultures is very different to STRs and is achieved via diffusion. It is also dependent on the surface to volume ratio, and as oxygen is sparsely soluble, the aeration in these systems becomes limiting. Randers-Eichhorn *et al* (1996) conducted mass transfer studies and on-line measurement of dissolved oxygen using optical sensors and found that the DO concentration at the bottom of T-flasks reached zero during cell growth, indicating the poor mass transfer in T-flasks. Oxygen depletion leads to non-optimal growth and ultimately cell death. Randers-Eichhorn *et al* showed that after only 2 passages, cells were exposed to anoxic conditions of DO below 0.5% for 18.5hours (per passage). Cyclic exposure to anoxic conditions can be detrimental for the cells as it can decrease the level of antioxidant molecules, sensitising cells to oxidant damage (Randers-Eichhorn *et al*, 1996).

Stirred tank reactors, as used in the current study, are able to produce significantly higher mixing and mass transfer values than the static cultures used in the literature. With the addition of online DO sensors in STRs, a constant DO level can be achieved, using feedback control with oxygen sparging. Therefore, there are significant differences in the dissolved gas concentrations between the vessel applied in this study and those used in literature sources with the differing results.

In addition to the differences in DO levels, the lack of mixing in static cultures leads to non-ideal and uneven distribution of the liquid and gas phases, which results in nutrient/by-product, dissolved gasses and pH gradients and these gradients are frequently blamed for reduced productivity (Xing *et al*, 2009). Culture pH, nutrient availability and dissolved gasses significantly affect cell growth and metabolism. DO and pH have shown to affect the cell growth, recombinant protein production and glycosylation profile. Therefore, these process parameters have to be optimised and controlled to ensure good process performance (Yoon *et al*, 2005; Trummer *et al*, 2006). As the control of these parameters is not possible in static and micro-scale vessels, significant differences can occur compared to bench scale STRs (as used in this study), therefore, the results from the differing system types cannot be compared. The best performing clones in small scale devices often do not translate to the larger scale reactors, indicating that the operation of these systems are not scalable (Nienow, 2015).

A number of previous studies have not considered the interactions between un-optimised controls or process parameters, such as the decoupling of pH and pCO_2 control, and process intermediates

(e.g. ammonium and lactate). For example, studies have shown an effect between bicarbonate concentration with osmolality and pH under elevated pCO_2 (Zanghi *et al*, 1999) and the interacting effect of ammonium with pH level on cell culture processes (Val *et al*, 2010). Inherently, the cell metabolism is multivariate with a number of factors having an effect. Therefore, if in-line/at-line monitoring of parameters is not present, then the source of interacting effects cannot be identified. Since the publication of the majority of studies, a number of advances in the in-line/at-line monitoring and controls have occurred, such as improved inline sensors and at-line analytics. A lack of process monitoring and controls in the small scale systems will inherently lead to differences in the culture system, resulting in multivariate interacting effects, such as effects from pH and DO. In a number of previous studies, feedback control (as used in the current study) was not applied, resulting in poor control of pCO_2 levels (deZengotita *et al,* 1998; deZengotita *et al,* 2002; Gray *et al,* 1996; Kimura and Miller, 1996; Zhu *et al,* 2005).

It has been reported that pCO_2 and/or osmolality level had a negative or limited impact on the productivity of a mammalian cell culture (deZengotita *et al,* 1998; Gray *et al,* 1996; Kimura and Miller, 1996; Zhu *et al,* 2005). It should be noted that in these studies STR's were not used to generate experimental data nor were engineered scale-down tools described. Instead, the authors reported data generated in static T flasks or agitated micro-well plates, which for the reasons given above (Section 1.3.1) are not fully representative of the performance of an STR. As a consequence, a number of process parameters, including pH and DO (which are known to interact) were not optimised, resulting in sub-optimal conditions before additional stresses were introduced to the system (deZengotita *et al,* 1998; deZengotita *et al,* 2002; Gray *et al,* 1996; Kimura and Miller, 1996; Zhu *et al,* 2005).

Gray *et al* (1996) observed a 50% decrease in the relative specific productivity of a perfusion culture with an increase in pCO_2 from 35 mmHg to 148 mmHg. The main cause of the decrease in productivity was due a decrease in cell titre and culture viability, from 79% to 58% at 35 and 148 mmHg, respectively. However, it was hypothesised that this is due to the fact that the perfusion culture at the control condition was below normally accepted values (below 95% viability), indicating a non-optimised process before additional stresses were added.

Kimura and Miller (1996) and deZengotita *et al* (1998 & 2002) utilised T-flasks and well plates to carry out elevated pCO_2 studies. As discussed previously, T-flask operation and performance are not scalable or comparable to STRs, which is the traditional industrial reactor type that was used in the current research. In the study conducted by deZengotita *et al* (1998), a significant difference in specific productivity relating to pCO_2 control was not observed. This was due to the presence of

significant variation in measured responses between repeats under the same conditions, which may have been due to a lack of control when using T-flasks. When experiments were repeated in STRs, under elevated pCO_2, a 70% greater specific antibody production rate was observed, which agrees with the results obtained in the current study, but there was limited discussion on the origin of this high production rate in their paper. The authors commented that caution is required when obtaining results from different culture systems.

Zhu *et al* (2005) examined elevated levels of pCO_2 in STRs utilising a comparable system to the ones used in the current study, reporting that elevated pCO_2/osmolality had an adverse effect on process performance. However, Zhu *et al* (2005) did not decouple pCO_2 and pH control; instead adjusted the pH with the addition of bicarbonate to modify the pCO_2/pH equilibrium. Therefore, there may have been an interaction between the pH and pCO_2 control and bicarbonate addition. Additionally, pH may have varied significantly from the formation of metabolism by-products, which would affect process performance between the CO_2 control levels. Zanghi *et al* (1999) reported that bicarbonate concentration and pH/pCO_2 interactions have a negative effect on cell culture performance. This highlights the need to consider interacting effects whilst examining other process parameters, due to the multivariate nature of cell culture processes.

A number of studies have reported increased cell specific protein productivity at increased pCO_2/osmolality, but not overall titre, due to a drop in cell growth (Ozturk and Palsson, 1990; deZengotita *et al,* 1998; Brady, 2009). This is contrary to the results observed in the current study, where an increase in cell specific protein productivity and overall titre were observed. These differences are likely to be due to the differences in the culture system used (T-flask/well plates versus STRs), as discussed previously.

Ozturk and Palsson (1990) and deZengotita *et al* (1998) reported that specific antibody production for hybridoma cells increased with increased osmolality, as was observed for increased pCO_2 levels in the current study. However, due to lower growth and increased death rates observed by Ozturk and Palsson (1990) and deZengotita *et al* (1998), the overall mAb titre did not increase. In the present study, overall product titre and productivity increased with elevated pCO_2 control and the associated osmolality increase. This suggests an interacting factor between pCO_2, osmolality and other process parameters, which may be due to the difference in vessel type and process controls (STR versus T-Flask/well plate) or due to cell line specifics.

Eisenkraetzer *et al* (2013) reported that elevated pCO_2 control resulted in higher productivity and product titre, as observed in the current study. However, the higher product titre was due to a

slower decline in cell culture viability and at equivalent time points the uncontrolled batch had a similar titre to the higher pCO_2 condition. Although osmolality was controlled in their study, the uncontrolled low and high pCO_2 conditions did exhibit differences in osmolality values, greater than 20 mOsmo.Kg^{-1}. The highest titre obtained by Eisenkraetzer et al (2013) was when using a dynamic profile of 5 to 15% pCO_2 (i.e. the value was not kept constant), which was designed to mimic pCO_2 enrichment in the culture medium during the fermentation process in industrial production processes.

The effect of elevated pCO_2 control on productivity may be cell line specific, as suggested by previous studies, hence the differences observed in this study. However, more importantly the interactions of other process and control parameters with pCO_2 control appear to play a significant role on the cell culture process. In studies where experiments were carried out in both well plates and STRs (deZengotita et al, 1998), significant differences in performance were noted, with an improved performance in STRs. The difference in results for different vessels is likely due to the increased process controls in STRs compared to the well plate systems. Therefore, along with the pCO_2 control level, the optimisation of other process parameters, such as the control of pH and DO alongside pCO_2 control, is essential due to interacting effects and the multivariate nature of cell culture systems.

The mechanism for the observed increase in productivity at elevated osmolality/pCO_2 is poorly understood. A number of theories have been proposed for the increase observed at elevated pCO_2/osmolality, including enhanced nutrient transport into the cell, increased mass per cell, cell cycle arrest in a more efficient stage, increased transcription/translation rates and alteration of the pH_i (Brady, 2009; Eisenkraetzer et al, 2013; Silva et al, 2013; Lin et al, 1999). This lack of understanding is due to the difficulty of analysing cell culture systems, particularly the cell metabolism, as a consequence of the multivariate nature of the cell and the fact that the internal cell metabolism is difficult to quantify. Additionally, it is a challenge to analyse the response of a single factor in a cell culture process, due to the highly interlinked/multivariate nature of the cell metabolism. More specifically, only a fraction of the possible parameters are currently measured and a change in one factor influences a number of other factors, which, in turn, can influence the cell culture performance. Measurements of the pH_i and internal cell metabolism are possible, however, these are offline methods and require a large number of assumptions and modelling techniques (Dean and Reddy, 2013; Duarte et al, 2014; deZengotita et al, 2002; Templeton et al, 2013). The likely cause of the increased productivity is considered to be the effect of pCO_2/osmolality on the pH_i and nutrient transport, as previous studies have reported that pCO_2/osmolality affects these factors,

which, in turn, would affect the cell metabolism. The precise cause of the effect of pCO_2 control is not known and to determine this, advances in the measurement and modelling of the cell metabolism is required. However, a number of possible causes have been proposed in the literature as summarised below (Brady, 2009; Eisenkraetzer *et al*, 2013; Lin *et al*, 1999; Silva *et al*).

A number of studies have suggested that an increase in q_{mAb} observed at high osmotic pressures is due to increased flux of amino acids and nutrients into the cell (Brady, 2009; Lin *et al*, 1999). Brady (2009) reported a dose response in the internal concentration of amino acids and the derivatives under elevated osmolality and Lin *et al* (1999) concluded that higher specific ATP production rates from carbon sources were associated with flux changes under high osmotic pressure.

Eisenkraetzer *et al* (2013) and Silva *et al* (2013) stated that elevated pCO_2/osmolality affects the pH_i of a mammalian cell. Eisenkraetzer *et al* (2013) specified that elevated pCO_2 levels increase pH_i, which leads to the intensification of the oxidative metabolism (with the conversion of pyruvate to oxaloacetate for corporation into the TCA cycle).

Both Eisenkraetzer (2013) and Silva *et al* (2013) reported that elevated pCO_2 levels triggered quicker progression of cells into the G1/G0-phase of the cell cycle, reportedly a more productive cell stage when coupled with increased pH_i. Therefore, elevated production levels may be due to the effect of pCO_2 on pH_i and hence the cell metabolism. However, studies showed contradicting results on the effect of pCO_2 on pH_i, demonstrating that cell line or process specifics have an effect (deZengotita *et al*, 2002; Eisenkraetzer *et al*, 2013; Silva *et al*, 2013). deZengotita *et al* (2002) reported a decrease of the hybridoma cell pH_i when pCO_2 was increased. In contrast, Eisenkraetzer *et al* (2013) and Silva *et al* (2013) reported that elevated CO_2 leads to an increase in pH_i. These contradicting results suggest that cell line specifics or other interacting process parameters, such as culture pH, play an important role in the mammalian cell metabolism.

In summary, the results presented in this study demonstrate that by increasing pCO_2 control level, with an associated osmolality increase, an increase in cell specific productivity and overall product titre is achieved. These results are in contrast to a limited number of previous studies and are likely to be due to differences in mixing/mass transfer and process monitoring and controls, including vessel and media type, leading to interacting effects from other parameters, such as pH, DO and cell metabolism by-products. The cause of the increased productivity is unclear, whether pCO_2, osmolality or an interaction between both parameters is responsible. It is likely that increasing pCO_2 control does have an effect on pH_i, which can influence both the cell metabolism and gene regulation (Eisenkraetzer *et al*, 2013; Silva *et al*, 2013). However, measurement of the pH_i and amino

acid fluxes during the cell culture process is required to determine if they play a role in increased productivity.

2.4.2 Product Quality

Elevated pCO_2 at 140 mmHg showed a similar target mAb composition compared to the baseline for charge variants analysis. Increasing the pCO_2 control from 20 to 100 mmHg resulted in a reduced target mAb composition compared to the baseline condition.

There has been limited literature published regarding the effect of elevated pCO_2 and osmolality on the product quality attributes of monoclonal antibodies (Table 2.6). Previous studies concluded that an elevated pCO_2 level had limited impact on the product quality attributes of a protein product when examining the glycosylation of a CHO derived tissue plasminogen activator (tPA) and the charge distribution and monosaccharide content of a mAb (Kimura and Miller, 1997; Schmelzer and Miller, 2002).

The results presented from the current study differ to those reported in the literature (Kimura and Miller, 1997; Schmelzer and Miller, 2002) However, it is difficult to make comparisons as different product quality attributes were examined in the previous studies compared to the current study. The differences are considered to be due to cell line and process control variations, i.e. the use of stirred tank reactors with full pCO_2 and additional process control compared to static T-flask cultures with environmental pCO_2 control (as mentioned in the previous section). Additionally, differences in the results reported in the literature and this study are likely to be due to sub-optimal process controls and conditions or media and cell line specifics used in the previous studies. This highlights the need to optimise the process conditions alongside pCO_2 control. Studies on the effect of pCO_2/osmolality on mAb product quality were carried out over 10 years ago. Therefore, advances in process monitoring and control, media formulation, cell lines, analytical techniques and a large increase in product titres, may have also played a role in the varied results observed in the current study.

Product Quality Attribute	Process Purity Attributes
Aggregation	Microbiological Purity
Charged Variants	Viral Purity
Conformation	DNA
C-Terminal Lysine	Host cell protein
Deamidated Isoforms	Cell culture medium component
Disulfide Bonds	
Fragmentation	
Glycation	
Glycosylation Oxidation	

Table 2.6 - Typical Quality Attributes for a Monoclonal Antibody (CMC Biotech Working Group, 2009)

Little difference in the measured variables between the pCO_2 levels was observed in the current study to account for the effect on product quality. The most significant difference measured was the ammonium and lactate metabolism (Figure 2.9 (b) and (c)). Increasing pCO_2 levels from 20 to 100 mmHg resulted in increased levels of ammonium, with reduced levels at 140 mmHg compared to the baseline. Previous studies have demonstrated that ammonium levels can negatively affect the product quality (Gawlitzek *et al*, 2000; Chen and Harcum, 2006). A number of other studies have suggested that increased ammonium levels elevate pH_i, inhibiting glycosylation (Chen and Harcum, 2006; Gawlitzek et al, 2000).

It is possible in the results presented in this study that improved product quality is due to decreased ammonia levels at the baseline and 140 mmHg conditions compared to 20 to 100 mmHg conditions. However, it was observed that, the ammonium concentration at 140 mmHg was double that of the other conditions (which have similar values), at the initial time point for charge variants (144 hours). Therefore, it is probable that other interactions play a role alongside ammonium. An example of this is shown by Val *et al* (2010) where ammonium and pH were shown to have a synergic effect on protein N-glycosylation macro-heterogeneity.

A possible mechanism for the improved quality at elevated pCO_2 is the effect on the pH_i, suggested by the lack of significant differences in the measured variables. Carbon dioxide is a non-polar molecule, which can easily diffuse through the cell membrane and enter the cytosol and mitochondrial compartment of the cell. Once inside the cell, CO_2 forms carbonic acid, protons and bicarbonate atoms, which affect the pH_i, which, in turn, directly influences cellular processes and pH sensitive enzymes. Although little work has been carried out looking at the effect of pH_i on mammalian cell processes, a number of investigations were undertaken examining the effects of external pH (i.e. culture pH). Previous studies have demonstrated that the manipulation of culture pH affects productivity and product quality (Larsson *et al,* 2008; Rendall *et al*, 2005; Trummer *et al*, 2006; Yoon *et al,* 2005).

2.4.3 Cell Growth

A number of studies have shown that cell growth is inhibited under elevated osmolality and pCO_2 (Gray *et al*, 1996; Kimura and Miller, 1998; Zhu *et al,* 2005). In these studies, osmolality was shown to have the greatest effect on cell growth with pCO_2 being of less importance. Therefore, the

reduced growth rate and maximum cell titre observed in the current study are possibly due to the combined effect of elevated pCO_2 and osmolality, with osmolality playing a more significant role.

The cause of the inhibitory effect of elevated pCO_2/osmolality on cell growth is not fully understood, with a number of studies contributing the decrease to osmolality and/or pCO_2 (Gray *et al,* 1996; deZongotita *et al*, 1998; Kimura and Miller, 1996). Possible theories on the impact of pCO_2/osmolality include changes to the pH_i, which affects pH dependant processes within the cell, and the direct effect of elevated pCO_2/osmolality levels on the cell metabolism.

As opposed to the findings of a number of previous studies (Ozturk and Palsson, 1990; deZengotita *et al*, 1998), in this study the decreased cell titre did not negatively impact on the final mAb titre. This effect may be due to cell line and process differences; however, it more likely demonstrates that optimisation is required for pCO_2/osmolality levels and control along with other interacting process parameters, which were not considered in the previous studies.

2.4.4 Cell Metabolism

Analysis of the media amino acids gives an indication of the cell metabolism in terms of extracellular amino acids. Analysis of the internal amino acid metabolism is not available without metabolic flux analysis. The metabolism of glutamine, ammonium, glucose, lactate and alanine are important in terms of the cell culture and are inter-related. The metabolism of these amino acids displayed the most significant change as a result of pCO_2 control level.

In summary, the study results demonstrate that glutamine (in the form of L-alanine-L-glutamine) metabolism is strongly linked to the lactate and alanine metabolism, which is, in turn, linked to the ammonium metabolism along with glutamate and glycine, Figure 2.16 and Figure 2.17, which supports what has been reported in the literature (Dean and Reddy, 2013; Li *et al*, 2012; Zagari *et al*, 2013).

The results reported in this study agree with previous investigations into the effect of elevated pCO_2/osmolality on lactate and ammonium, with an increase observed with increasing pCO_2/osmolality (Ozturk and Pallson, 1990; Schmelzer and Miller, 2002; Zhu *et al*, 2005). However, previous investigations did not show a decrease or shift in ammonium production with elevated pCO_2 (140 mmHg), which is likely due to cell line/process specifics. In this study, a reduction in ammonium levels was observed at the highest pCO_2 level, which has not been reported in the literature. Additionally, in this study, a shift in the lactate and ammonium profile was observed at the highest pCO_2 level, shown in Figure 2.9 (b) and (c).

Increasing pCO_2 control led to an increase in maximum lactate levels, during exponential growth. This increase in lactate levels is likely to be due to a combination of the glutamine source (Glx) metabolism and the reduced growth rate with increasing pCO_2 control. At reduced growth rates, the demand for cell growth associated components from the TCA cycle is reduced. As a result, a greater amount of glutamine can be converted to lactate through the TCA cycle (via glutaminolysis), during the exponential phase. It is also possible that the increased lactate levels result from the increased flux of lactate out of the cell, due to the difference between the pH_i and external pH. This theory has been discussed as a cause for the flux of lactate out of the cell by Eisenkraetzer (2013), which may be due to re-establishing a pH equilibrium. However, analysis of pH_i and metabolic flux analysis is required to determine if differences in the pH_i is the cause of increased lactate at increased pCO_2.

A delay in the switch in the lactate metabolism from production to consumption was noted at the highest control levels (100 and 140 mmHg). The switch in lactate metabolism coincides with the depletion of Glx (the glutamine source) and Asn, for all conditions, which, in turn, corresponded with the switch from exponential to stationary cell growth. Glutamine metabolism has previously been linked with the production of the majority of lactate during the exponential growth phase (Dean and Reddy, 2013; Li *et al*, 2012). Asn depletion has also been linked with the arrest of cell growth (Duarte *et al*, 2014). During the growth phase, glutamine is rapidly consumed, as a key source for the TCA cycle and converted to lactate (and alanine), via pyruvate, by a process known as glutaminolysis. This process produces NADPH, which is used in anabolic processes required for cell growth, hence the lactate build-up to supply the reducing power (i.e. NADPH) for growth (Zagari *et al*, 2013).

There is evidence for the depletion of glutamine (and Asn) as the cause of the switch in lactate metabolism through the observation that the delay in Glx/Asn depletion at 140 mmHg corresponds to a delay in the re-metabolism of lactate compared to the other conditions shown in Figure 2.16. This study suggests that Asn is also linked to the shift in the re-metabolism of lactate, as it is depleted at a similar rate to Glx. Therefore, the results suggest that operation at elevated pCO_2 control delays the switch from the exponential to stationary cell growth and also the metabolism of lactate, through the limitation of the Glx and Asn metabolism.
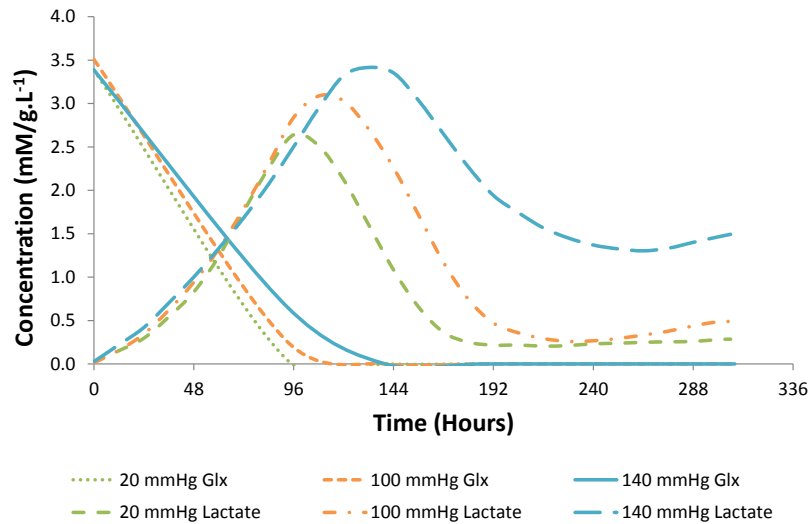
Figure 2.16 - Glx (mM) and Lactate(g.L^{-1}) Metabolism for the 20, 100 and 140 mmHg Conditions (Showing the delay in Glx depletion leading to a delay in the switch in Lactate metabolism)

Compared to the baseline condition, ammonium concentrations at 140 mmHg were lower. The likely cause of this is the increased lactate and reduced alanine levels at 140 mmHg. This is a result of lactate being a preferred carbon source, which substitutes the catabolism of certain amino acids that cause the formation of ammonium, such as alanine (Dean and Reddy, 2013; Li *et al*, 2012; Zagari, 2012). Therefore, at 140 mmHg, as the consumption rate of lactate is increased, the consumption rate of alanine is decreased, reducing the formation of ammonium.

At 20 to 100 mmHg, the ammonium concentrations were greater than for the baseline conditions. The lower harvest ammonium concentration in the baseline is likely due to less active cells (TCA cycle) in the baseline, as indicated by the reduced mAb production and decreased consumption rate of amino acids, including glutamate and glycine. Alanine is consumed from 120 to 192 hours, which coincides with the switch from ammonium consumption to production. In the baseline condition, once alanine is consumed, both the mAb (Figure 2.1) and ammonia production reduce and deviate from the 20 to 100 mmHg conditions, suggesting a drop in the cell/TCA activity. At 20 to 100 mmHg, ammonium is produced at a higher rate, as other amino acids (such as glutamate and glycine) are catabolised, leading to the formation of ammonium and deviation in ammonium levels post 192 hours.
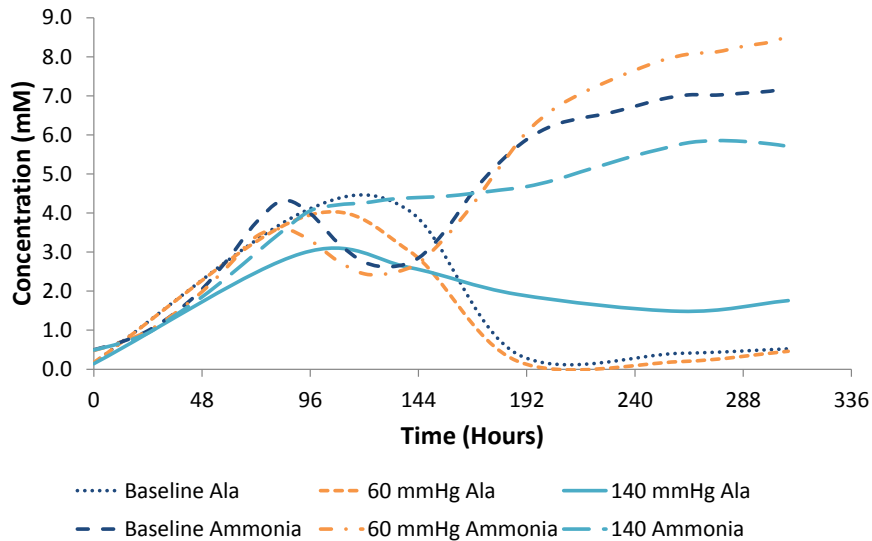
Figure 2.17 - Mean Alanine and Ammonium Profiles for the Baseline, 100 and 140 mmHg Conditions

Figure 2.17 demonstrates the link between alanine consumption and ammonium production. As alanine is consumed from 120 to 192 hours, the ammonium metabolism switches from consumption to production. Post-192 hours, when alanine is no longer consumed, the production rate decreases (as other amino acids are catabolised producing less ammonium, such as glycine). At 140 mmHg, the alanine consumption rate is reduced (due to increased lactate levels), as is the ammonium production rate, whilst the consumption of other amino acids (such as glycine, glutamate) and lactate are higher than observed in the baseline. Therefore, it is hypothesised that elevated pCO_2 reduces ammonium levels in cell culture through the limitation of alanine metabolism, as a result of increased lactate levels during exponential growth. This agrees with the results reported in the literature that increased lactate reduces ammonium formation (Dean and Reddy, 2013; Li *et al*, 2012; Zagari, 2012). Figure 2.17 shows a strong negative correlation between alanine and ammonium post 120 hours.

It is likely that the alternative pathways to alanine consumption, such as glycine consumption, results in reduced levels of ammonium produced for the 140 mmHg conditions, as suggested by Chen and Harcum (2006), shown in Figure 2.18, of which glycine metabolism is an ammonium sink.
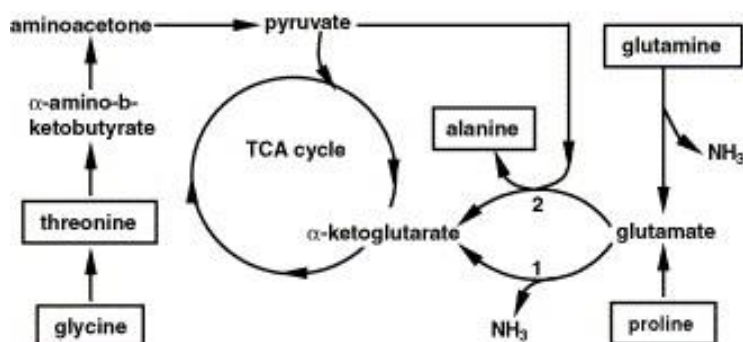
Figure 2.18 - Schematic Diagram of Amino Acid Metabolism for Alanine, Proline and Threonine as Related to Ammonium (Chen and Harcum 2006)

The cause and effect of the differing amino acid concentrations need to be understood to give an insight into the effect of pCO_2 control on the cell metabolism. However, a full metabolic flux analysis of the cell amino acids is required to fully understand the effects along with analysis of pH_i.

The cell metabolism profiles suggest that the TCA cycle, particularly during the production phase is more active in the pCO_2 control conditions compared to the baseline, indicated by the increased consumption of amino acids and production of ammonium and lactate. This is reflected in the greater mAb production and increased productivity for pCO_2 control levels compared with the baseline. This indicates that pCO_2 level impacts the cell metabolism; therefore, differences during operation and scale-up in pCO_2 are likely to affect process performance.

A significant advance in media formulation has occurred in recent years with the most significant change being the replacement of serum with serum-free, chemically defined medium. The composition of the medium has the most significant impact on cell culture process, including the micro nutrients, vitamins, antioxidants and trace metals which have a significant impact on cell culture data. By way of example Pall Corporation recently generated internal data between two media types which demonstrated a significant difference in cell culture performance, despite having almost identical amino acid content, with the cells reacting differently to the two media (Pall Corporation, personal communication).

2.5 Conclusions and Further Work

Increasing the pCO_2 control level from 20 to 140 mmHg led to increased productivity compared to the baseline condition for a mAb producing CHO cell line, with a 40% increase in product titre at the maximum pCO_2 level investigated (140 mmHg). Elevated pCO_2 control at 140 mmHg led to maintenance of product quality attributes (charge variants) at elevated productivity, whereas

operating at 20 to 100 mmHg led to a reduction in the quality attributes compared to the baseline condition.

Control of pCO_2 affected the cell metabolism in terms of cell growth and amino acid metabolism. At 140 mmHg, reduced ammonium concentrations were noted, due to increased lactate levels and reduced consumption of alanine. Additionally, increased consumption rates of glycine and reduced production rates of glutamate were observed during the stationary phase, compared to the baseline. This potentially indicates a more active TCA cycle with increased pCO_2 control level. In the baseline condition, post 192 hours, the TCA activity apparently dropped. This is indicated by a drop in mAb and ammonium production.

The results were not in agreement with the varied results reported in the literature on the effect of pCO_2/osmolality on a mammalian cell culture process. The majority of studies previously carried out were conducted over 15 years ago and since then, advances in process monitoring and control, media formulation, cell lines, analytical techniques and large increases in product titres, have occurred and these are likely to have played a role in the differences in results observed in the current study. In a number of the previous studies sub-optimal process controls and conditions were used, such as not decoupling pH and pCO_2 control and/or the use of unrepresentative reactor vessels, leading to interacting effects from other process parameters. Due to the multivariate and interlinked nature of the cell metabolism, a number of interacting factors play a role on the cell culture performance. This highlights the need to optimise/consider the process conditions alongside pCO_2 control.

The study results have indicated that pCO_2 control levels do affect a cell culture process, in terms of both productivity and product quality. Therefore, the pCO_2 control level should be considered in QbD, small scale and scale-up studies, as it will impact on process performance.

Further work is recommended through the monitoring of the pH_i and metabolic flux analysis (with ^{13}C labelling) to examine the effect of pCO_2/osmolality on the internal cell metabolism and pH. A number of methods have been described in the literature for the analysis of the internal pH and cell metabolism (Dean and Reddy, 2013; Duarte *et al*, 2014; deZengotita *et al*, 2002; Templeton *et al*, 2013). However, these methods cannot be performed inline/at-line and are costly, time consuming and require a number of modelling assumptions. Additionally, the analysis of pyruvate (intra and extracellular) would provide increased knowledge of the fate of a number of metabolites, including Glx, lactate and Ala, as pyruvate is a key component of the TCA cycle. Extracellular pyruvate can be

measured using commercially available assays; however, the analysis of internal pyruvate levels would be more beneficial, as this would give an indication of the internal cell metabolism.

Furthermore, for future applications the robustness and accuracy of pCO_2 inline and at-line sensors require improvement as they are prone to drift, particularly at higher concentrations. This would also allow in the determination of the operating space for pCO_2 control level for the process used in this study, as operation above 140 mmHg was not possible, due to limitations in the measurement range of the inline and at-line analytics.

3 Effect of Carbon Dioxide Control and Osmolality Level on a Mammalian Cell Culture Process

3.1 Introduction

An osmolality increase was observed with increasing pCO_2 control level, due to the increased base addition and differences in metabolism (e.g. higher lactate levels). Review of literature had demonstrated that osmolality can affect a cell culture process. Before the start of the study, it was hypothesised that the osmolality increase was not significant enough to affect the performance of the process, as deZengotita (1998) had shown an effect with large increases in osmolality, of 150 – 250 mOsmo.Kg^{-1} units. In this study, the difference between each condition was a dose increase of 10-15 mOsmo.Kg^{-1}, with a maximum difference of 60 mOsmo.Kg^{-1} across the range (from the baseline to the 140 mmHg condition). In order to determine if the osmolality increase observed in this study had a significant impact on the process performance, a custom FortiCHO medium was used with a reduced osmolality to eliminate osmolality as a factor.

Initially a batch examining low pCO_2 control (20 mmHg) at low and elevated osmolality was carried alongside a 140 mmHg condition and a baseline condition.

The results from the initial study indicated that osmolality had a greater impact on the process performance than initially hypothesised. Therefore, a second study was carried out decoupling osmolality and pCO_2 control with the use of a custom medium formulation (using the same FortiCHO medium with a lowered osmolality by removing the sodium chloride from the formation, which is added to increase osmolality of the medium).

In this Chapter, the two studies examining the role of osmolality have been combined.

3.2 Materials and Methods

3.2.1 Cell Line and Medium

Details for the cell line and medium are provided in Chapter 2, Section 2.2.1.

3.2.2 Bioreactor Cell Culture Runs

Details for the bioreactor cell culture runs are provided in Chapter 2, Section 2.2.2.

3.2.3 pCO₂ Control and Osmolality Levels

Table 3.1 summarises the pCO_2 control levels carried out in the study. For each batch of STRs (Table 3.2), a baseline condition (i.e. no pCO_2 control) was carried out to normalise the data to account for batch-to-batch variation. A pCO_2 of 20 mmHg was selected as a condition to elevate the osmolality as it had a similar osmolality to the baseline. The aim of this was to be able to compare the effect of pCO2 and osmolality at high and low levels, along with high and low osmolality levels with no pCO_2 control. In this way, by comparing against the baseline, the effects of low and elevated pCO_2 and low and elevated osmolality could be assessed.

Factor	Levels	Description
CO₂ (mmHg)	3	Baseline, 20 & 140 (±10)
Osmolality Level	3	Baseline, Low, High

Table 3.1 - STR Control Conditions.

For the study, three osmolality levels, baseline, low and high and three pCO_2 conditions, baseline, low (20 mmHg) and high (140 mmHg) were selected. The baseline osmolality and pCO_2 refer to the starting level and time profile which occurred in the baseline process (i.e. unmodified and uncontrolled osmolality and pCO_2).

High osmolality refers to a similar osmolality as the standard 140 mmHg condition (Chapter 2), a starting osmolality of 375 ± 5 mOsmo.Kg^{-1}. Osmolality control levels were adjusted manually using a NaCl feed in order to match the desired osmolality level. The low osmolality condition refers to a similar osmolality as the 140 mmHg condition with a custom media in which the osmolality had been reduced, by removal of NaCl salt from the formulation, a starting osmolality of 330 ± 5 mOsmo.Kg^{-1}. NaCl is added to media formations for the purpose of increasing the media osmolality to the desired formulation.

Due to a difference in the time-dependent trend in osmolality between conditions, a 1M NaCl feed was used to match the osmolality to the desired control level. During the course of the 140 mmHg conditions, increased NaOH (base) addition was required to offset the increase in pCO_2, compared to the baseline and 20 mmHg conditions. Therefore, the NaCl feed was added to the baseline and 20 mmHg conditions to match the corresponding 140 mmHg control level. The average osmolality level for the test conditions is shown in Figure 3.1, which demonstrates that a constant osmolality was not maintained during the cell culture as solute particles cannot be removed from the media in a batch process. This effect was sustained following an additional glucose feed partway through the cell culture.
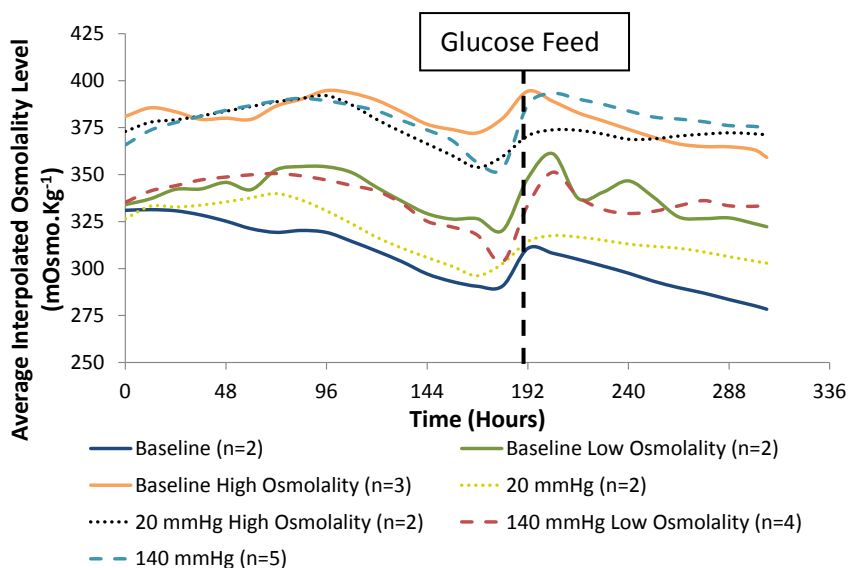
Figure 3.1 - Average Interpolated Osmolality Level during Cell Culture Runs

The STR conditions for each batch, including the condition which the osmolality control was matched to are shown in Table 3.2.

Test Batch	STR Run	pCO$_2$ Level	Osmolality Control (to match STR run)	n
1	1-1	Baseline	NA	1
	1-2	20 mmHg	NA	2
	1-3	20 mmHg	High (STR Run 1-4)	2
	1-4	140 mmHg	NA	1
2	2-1	Baseline	NA	1
	2-2	Baseline	Low (STR Run 2-4)	1
	2-3	Baseline	High (STR Run 2-5)	1
	2-4	140 mmHg	Low	2
	2-5	140 mmHg	NA	2
3	3-1	Baseline	Low (STR Run 3-3)	1
	3-2	Baseline	High (STR Run 3-4)	2
	3-3	140 mmHg	Low	2
	3-4	140 mmHg	NA	2

Table 3.2 - STR Conditions Examined in Each Batch, n indicates number of repeats

3.2.4 At-line Analytics

Details for the at-line analytics are provided in Chapter 2, Section 2.2.4.

Details for the offline analytics are provided in Chapter 2, Section 2.2.5.

N-linked glycan profiling was carried out by the analytics team at Pall Corporation, Portsmouth and determined by Hillic chromatography (H-Class Bio Acquity UPLC, Waters, Milford, USA) with detection by fluorescence (FLR) Quadropole time-of-flight mass spectrometer (Waters).

Offline Analytic	Assay Type	Instrument
Amino Acid Concentration	Reverse phase AccQ-tag™ derivatisation	UPLC
N-Linked Glycan Profile	Detection by fluorescence (FLR) Quadropole time-of-flight mass spectrometer	UPLC-FLR/QToF
mAb Charge Variants	Cation exchange	UPLC
Total mAb Concentration	Protein-A affinity	HPLC

Table 3.3 - Offline Analytics and Assay Type

3.3 Results

The baseline condition in the third batch was terminated early due to an agitator failure. Therefore, the baseline condition from the second batch was used for normalising batches two and three. The same passage number was used for both batches (second and third), which led to similar cell line performance, as indicated by the less than 1% difference between the baseline low osmolality conditions for batches two and three.

Comparison of the baseline conditions for this study to those of the previous study detailed in Chapter 2 showed comparable results (Appendix 8.3). Similar metabolism, cell growth, mAb and product quality trends were observed despite the clone line instability.

When interpreting results, caution is required as each control condition was not carried out within each batch. For example, the duplicate 20 mmHg high osmolality condition was only carried out in batch one, whereas all other conditions were carried out across at least two batches. Therefore, batch-to-batch variability will impact on other conditions but is not captured for the 20 mmHg high osmolality condition. Additionally, batch one was carried out at a higher passage number, which led to low levels of mAb being produced, which is consistent with the clone instability (which was discovered after batch one had been carried out).

3.3.1 Antibody Production

Antibody Titre

Increasing both pCO_2 control and osmolality level resulted in an increase in harvest mAb titre, compared to the baseline condition, as shown in Figure 3.2. Compared to the baseline condition, there was a 55% increase in harvest mAb at 140 mmHg, 36% at 140 mmHg low osmolality, 38% at 20 mmHg high osmolality, 14% at 20 mmHg, 26% at baseline high osmolality and 7% at baseline low osmolality levels.

The results indicated that both the increased osmolality level and the pCO_2 control level caused an increase in mAb titre. A 28-29% increase was observed when pCO_2 control was increased and osmolality level maintained and a 19-23% increase was obtained when osmolality was increased and pCO_2 control level maintained, across control conditions, shown in Figure 3.2.
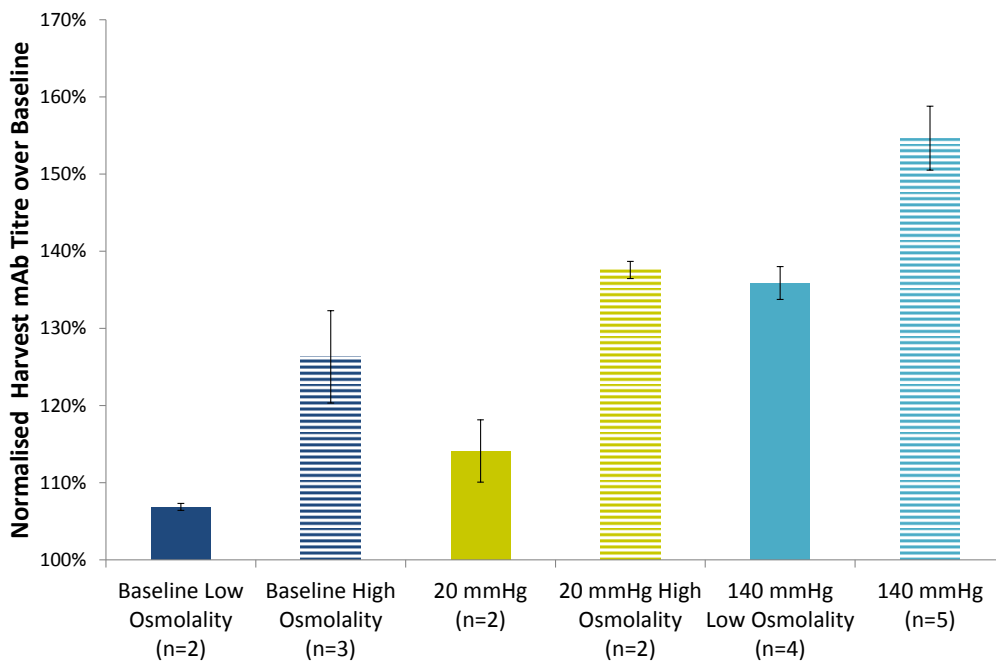
Figure 3.2 - Harvest mAb Titre Normalised over the Baseline Condition within Each Batch (Note: Shown with S.E. of the mean calculated using the normalised values. Solid bars were run at equivalent osmolality levels, controlled to match the 140 mmHg low osmolality condition and horizontal stripes were run at equivalent osmolality levels, controlled to match the 140 mmHg condition. The 20 mmHg condition had an equivalent osmolality level to the low osmolality conditions. The 20 mmHg high osmolality condition was harvested 12 hours earlier than other conditions as viability was at the lower limit of harvest density. The baseline conditions is not shown as the other conditions have been normalised against this condition)

Figure 3.3 and Figure 3.4 show the mean mAb production profile for all conditions, without normalisation against the baseline conditions. The difference in the baseline conditions between batches (i.e. Figure 3.3 compared with Figure 3.4) highlights the effect of passage number, as runs shown in Figure 3.4 were carried out at a higher passage number than those reported in Figure 3.3. Therefore, caution is required when analysing results between the batches, as the full effect of passage number for this cell line clone on the cell culture performance is currently not known with any certainty. The general trend indicates that both elevated pCO_2 and osmolality have an effect on product titre and, along with previous data (Chapter 2) that this overall trend is not affected by the clone line instability.
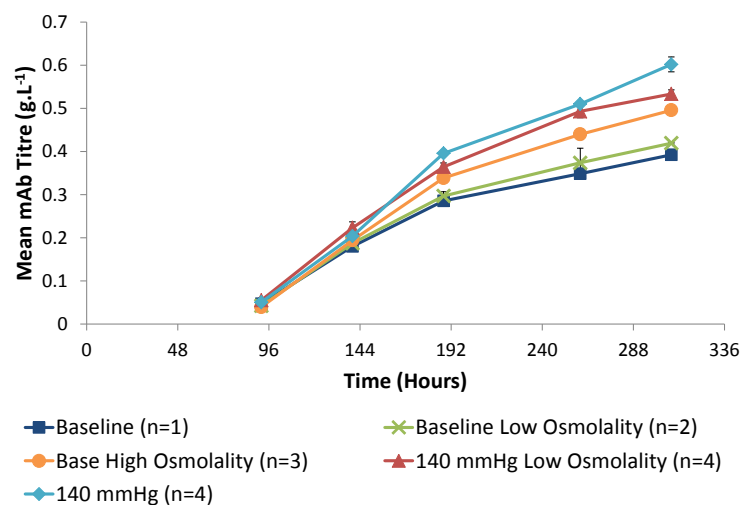
Figure 3.3 - Mean mAb Titre for Baseline, Baseline Low Osmolality, Baseline High Osmolality, 140 mmHg Low Osmolality and 140 mmHg Conditions (Note: Shown with S.E. of the mean)
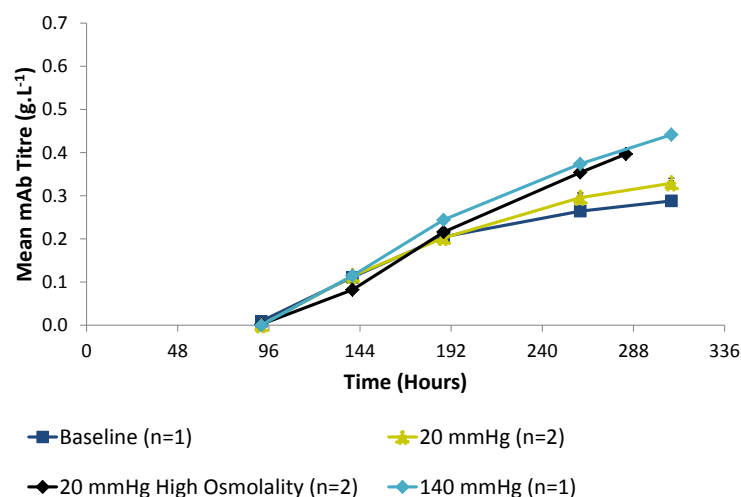
Figure 3.4 - Mean mAb Titre for Baseline, 20 mmHg, 20 mmHg High Osmolality and 140 mmHg Conditions (Note: The 20 mmHg high osmolality condition was harvested 12 hours early due to reaching the lower limit of the harvest density)

The 20 mmHg high osmolality condition resulted in lower final mAb titre due to a 12 hour earlier harvest titre, as the cell viability reached the lower limit of the harvest point (Figure 3.10). At the similar time points the 140 mmHg and 20 mmHg high osmolality conditions had similar mAb titres.

Cell Specific Productivity

The cell specific antibody production rate (q_{mAb}) is calculated using equation stated in Chapter 2.3.1, from the start of the stationary phase (144 hours) to the harvest point.
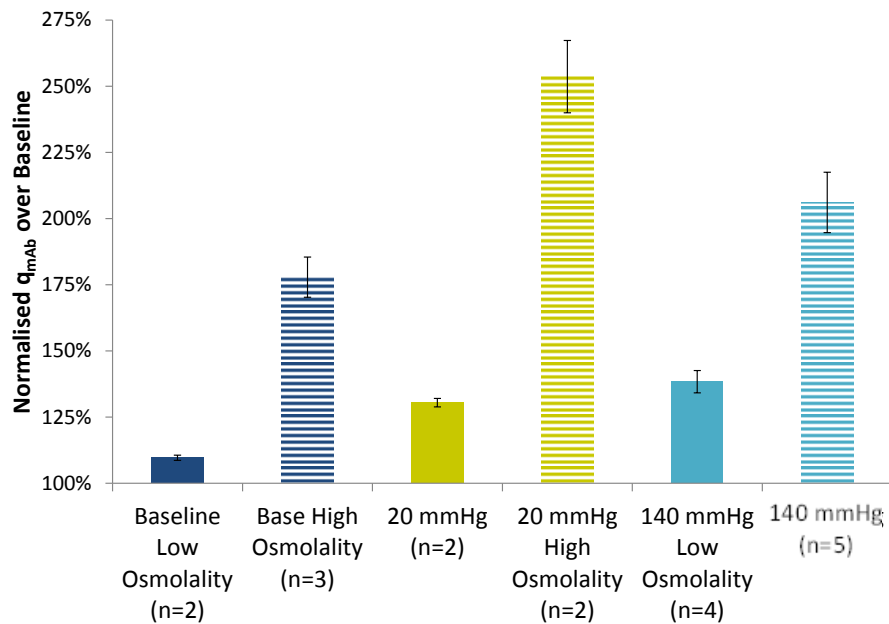
Figure 3.5 - Cell Specific Antibody Production Rate Normalised to the Baseline Cell Specific Productivity (Note: Shown with S.E. of the mean calculated using the normalised values. Solid bars were run at equivalent osmolality levels, controlled to match the 140 mmHg low osmolality condition and horizontal stripes were ran at equivalent osmolality levels, controlled to match the 140 mmHg condition. The 20 mmHg condition had an equivalent osmolality level to the low osmolality conditions. The baseline condition is not shown as the other conditions have been normalised against this condition)

Figure 3.5 demonstrates the effect of pCO_2 control and osmolality level on the cell specific production rate. The results appear to indicate that the 20 mmHg high osmolality condition results in the highest specific productivity. However, this is considered to be due to the 20 mmHg high osmolality conditions being carried out within one batch, so the effect of batch-to-batch variation was not captured. This batch was run using inoculum at a high passage number, which affects the mAb titre and cell growth. Additionally, the calculation of q_{mAb} is sensitive to cell counts, as it relies on a fit between the IVCD and mAb titre and errors in the cell counts transfers to the calculation.

Therefore, it can be considered that the 20 mmHg high osmolality and 140 mmHg conditions are not significantly different from each other.

The highest cell specific production rates were observed at the high osmolality conditions, with a 78%, 150% and 106% increase over the baseline condition for the baseline and 20 mmHg high osmolality and 140 mmHg conditions, respectively. A smaller increase in the cell specific production rates was noted for the lower osmolality level, with a 10%, 20% and 38% increase over the baseline condition for the baseline low osmolality, 20 mmHg and 140 mmHg low osmolality conditions, respectively.

3.3.2 Product Quality

Charge Variants

The charge variant profile has not been normalised against the baseline condition, as little batch-to-batch variation or variation due to passage number was seen in this or the previous study (Chapter 2).
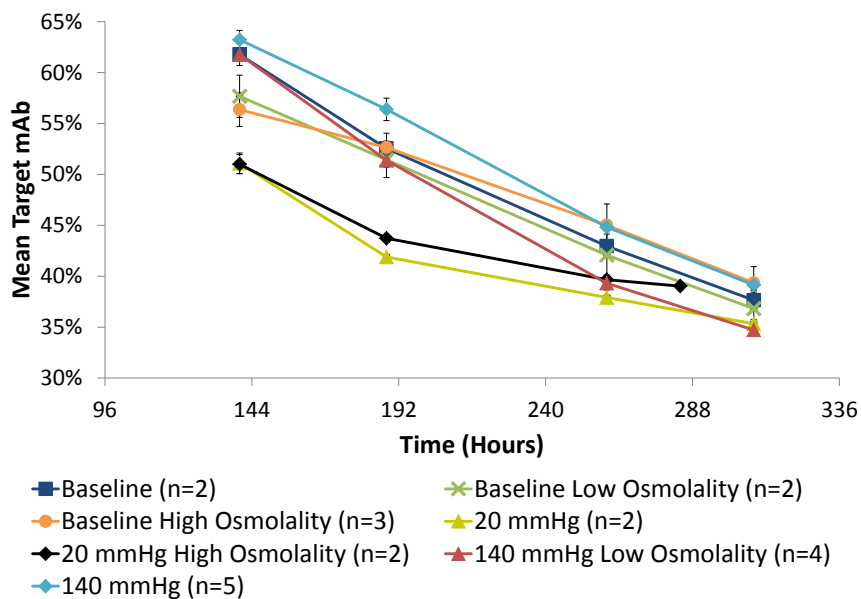
Figure 3.6 - Mean Target mAb Composition Time Profile of Charge Variants Analysis (Note : Shown with S.E. of the mean)

The target mAb profile is shown in Figure 3.6. The peak target mAb composition (144 hours) is 63% at 140 mmHg, 62% at the baseline and 140 mmHg low osmolality, 57% at the baseline low osmolality, 56% at the baseline high osmolality and 51% at 20 mmHg and 20 mmHg high osmolality conditions.

The general trend indicates that the 140 mmHg condition has the highest target mAb composition throughout the process. At the start of the stationary phase (144 hours), the baseline and 140 mmHg low osmolality conditions have similar target mAb compositions relative to the 140 mmHg condition, but degrade at a faster rate.

The baseline high and low osmolality conditions have a slightly lower (by 4-5%) target mAb composition relative to the baseline at 144 hours, the start of the stationary phase. At 144 hours, the 20 mmHg and 20 mmHg high osmolality conditions showed the largest decrease in target mAb, of 10%, compared to the baseline.

Throughout the culture, the target mAb decreased to a similar composition. At the harvest point all conditions were within 5% of each other, mainly due to the low cell viability. The major source of the degradation during the cell culture is a result of an increase in the acidic variants, which is reflected in Figure 3.7 and Figure 3.8(a), with minimal difference in the basic variant composition over time, Figure 3.8(b). Figure 3.7 indicates that the baseline and 140 mmHg conditions have the highest degradation rates of target mAb, with the slowest degradation in the 20 mmHg conditions.

The greatest decline in the target mAb was seen for the 140 mmHg low osmolality condition, which was 14% higher than observed in the baseline, Figure 3.7. There was less than 3% difference observed between target mAb gradient for the baseline and 140 mmHg condition (which is similar to the previous study, Chapter 2.3.2). An 11%, 28%, 38% and 51 % decrease in the target mAb gradient was seen for the baseline low osmolality, baseline high osmolality, 20 mmHg and 20 mmHg high osmolality conditions, respectively. Control of pCO_2 at 20 mmHg high osmolality indicates the lowest decline in target mAb, however, this is in part due to the earlier harvest for this condition, resulting in a 24 hr reduction in the time the mAb was exposed to the culture media.

Figure 3.7, indicates that the decrease in the gradient of the acidic variants between the baseline and 140 mmHg conditions was similar to the value observed in Chapter 2.3.2 (22%), with a value of 20%. A 10-17% reduction in the gradient, compared to the baseline, was observed for the baseline low osmolality, baseline high osmolality and 140 mmHg low osmolality conditions. The lowest decline in acidic variants was seen for the 20 mmHg conditions with a 32% and 51% decrease compared to the baseline condition. However, as discussed previously, the 51% decrease over the baseline observed for the 20 mmHg high osmolality condition is due to the earlier harvest time.
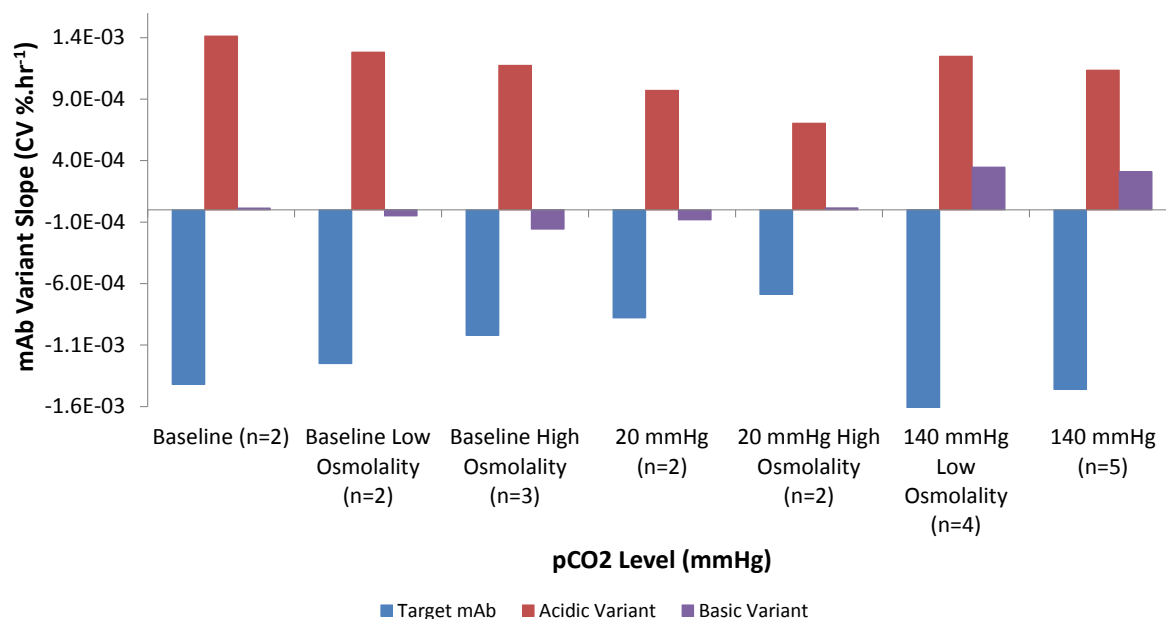
Figure 3.7 - Gradient of the Linear Fit of each mAb Variant during the Cell Culture

The lowest level of acidic variants throughout the culture was seen in the 20 mmHg high osmolality condition; but the highest level of basic variants was observed for this condition. The significantly higher levels of basic variants at the 20 mmHg and 20 mmHg high osmolality conditions are the cause of the decreased overall target mAb composition compared to the other conditions.

At the start of the mid-stationary phase (144 to 192 hours), the baseline condition, 140 mmHg and 140 mmHg low osmolality conditions have very similar basic variant compositions, as shown by Figure 3.8(b). However, the basic variants for 140 mmHg and 140 mmHg low osmolality increased at a slightly higher rate, with a difference of 4-5% by the end of the culture. The increase in basic variants for the 140 mmHg condition is countered by the lower levels of acidic variants compared to the baseline and 140 mmHg low osmolality, leading to an equivalent target mAb composition to the baseline condition at the harvest point (a 4% increase over 140 mmHg low osmolality). Therefore, despite the baseline and 140 mmHg conditions having the same target mAb composition, the acidic and basic make-up differ. This may affect the downstream processing of the product or the stability of the product in different ways.

As discussed in Chapter 2, the basic variants had minimal effect on the reduction of the target mAb, compared to the acidic variants, as shown in Figure 3.7. There was little difference in the gradient of the basic variants, with the greatest values seen for the 140 mmHg conditions.
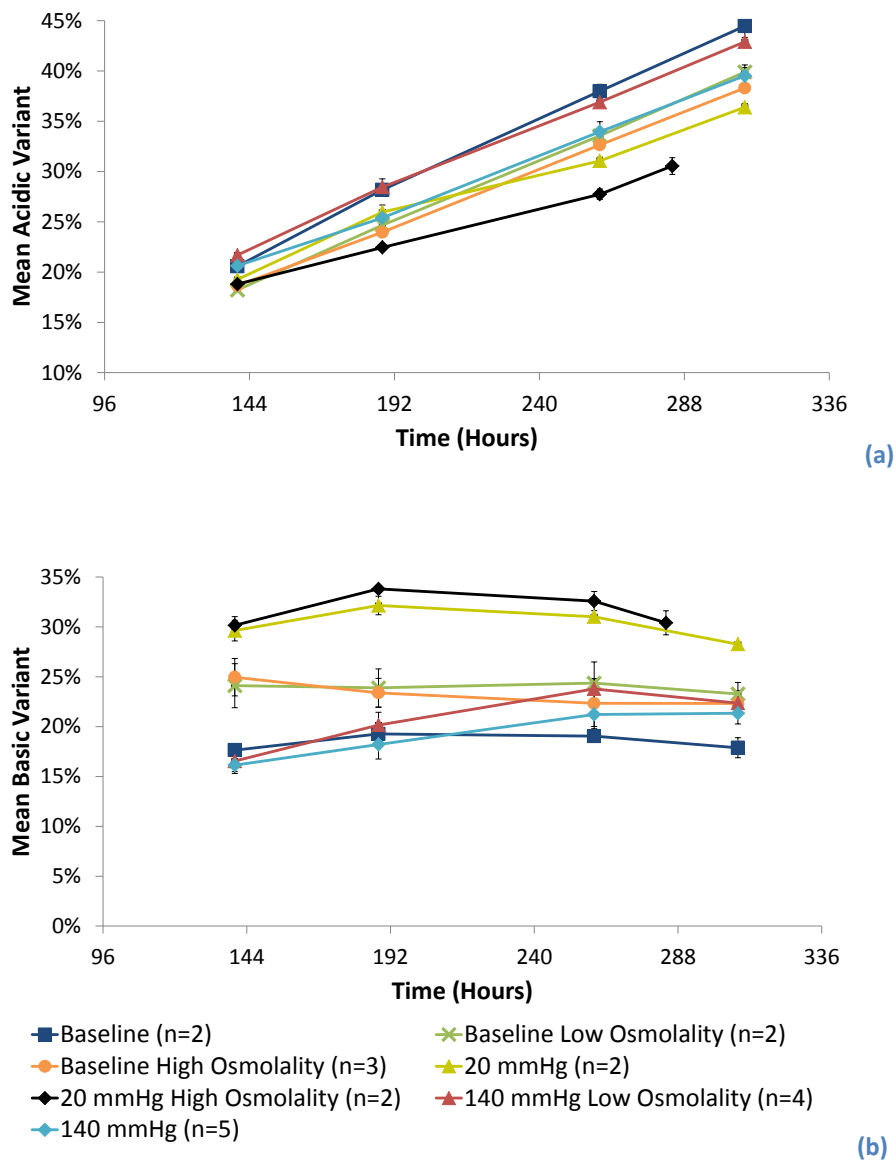
Figure 3.8 - Mean Acidic Variants (a) and Mean Basic Variants (b) Composition Time Profile (Note: Shown with S.E. of the mean)

The data suggests, in conjunction with the previous study (Chapter 2), that pCO_2 has the greatest effect on the charge variant composition, with an increase in basic variants with decreasing pCO_2 level. When pCO_2 is controlled, the osmolality level has little effect. When osmolality is adjusted to either low or high levels, there is an increase in the basic variants and a decrease in acidic variants.

N-Linked Glycan Analysis

N-Linked glycan analysis was performed on the harvest mAb samples of batch one (i.e. for the baseline, 20 mmHg, 20 high osmolality and 140 mmHg conditions). For the glycan analysis, the important feature is the glycosylation pattern as it can affect the safety and efficacy of the drug

product. Consequently, the aim in industry is to produce a comparable glycosylation pattern, i.e. a similar composition of each glycan (G0F, G1F etc.) relative to the desired form of the product, as determined in clinical trials. The N-linked glycosylation profile detected for all conditions are comparable to other mAbs published in the literature, with dominant peaks of G0F type minor structures of G1F, G2F and man-5 type (Berkel, 2009).

For the purpose of this study, the glycan pattern for the control conditions are compared against the baseline condition, as the desired glycan pattern for the mAb product used in this study has not been established.
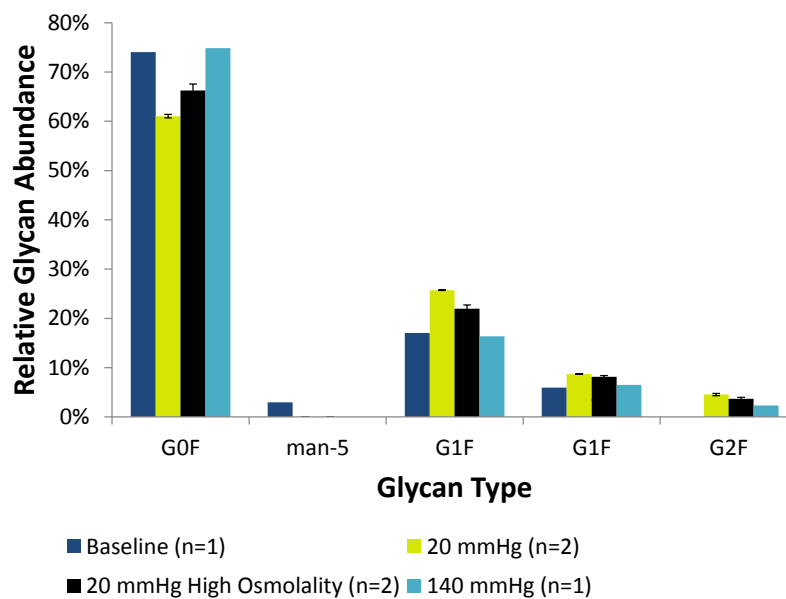
Figure 3.9 - Relative N-Glycan Abundance for Harvest mAb of Batch One (Note: The two G1F Glycan types listed are isomers of each other)

Figure 3.9 indicates that the baseline and 140 mmHg conditions have a similar glycan profile. The main difference between the baseline and 140 mmHg conditions is the presence of a man-5 peak and the absence of a G2F glycan in the baseline condition. The abundance of man-5 and G2F is less than 3% in the baseline and 140 mmHg conditions.

The 20 mmHg and 20 mmHg high osmolality conditions display similar profiles, which differ from the baseline condition, mainly due to an increase in G1F and a reduction in G0F abundance. Compared to the baseline, there is a 13% and 8% reduction in G0F for the 20 mmHg and 20 mmHg high osmolality conditions, respectively. The differences are due to an increase in the G1F (both forms which are isomers) and G2F for the 20 mmHg and 20 mmHg high osmolality conditions compared to the baseline.

3.3.3 Cell Growth

Viable Cell Growth Profile and Maximum Cell Titre
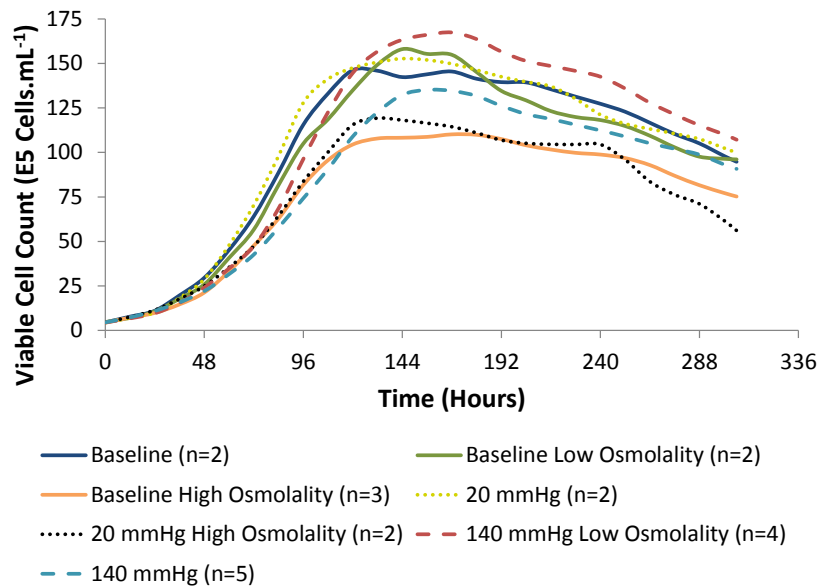
Figure 3.10 - Time Profile of Mean Viable Cell Growth for pCO₂ Control and Osmolality Levels (Note: data points have been interpolated to new equal time points by linear interpolation to determine means values)

A general trend of reduced maximum cell titre and growth rate at high osmolality conditions compared to the lower osmolality condition (at the same pCO_2 control levels), is shown in the growth curves in Figure 3.10 and maximum cell titre in Figure 3.11. Between conditions where the osmolality level was increased and the pCO_2 control level maintained (e.g. 20 mmHg versus 20 mmHg high osmolality) there was a 21% to 24% decrease in maximum cell titre. 7

The lowest cell growth was seen in the baseline high osmolality and 20 mmHg high osmolality conditions and the 140 mmHg condition, compared to the baseline condition. The highest cell growth was observed at the 140 mmHg low osmolality condition and an equivalent growth to the baseline condition was noted for the baseline low osmolality condition.
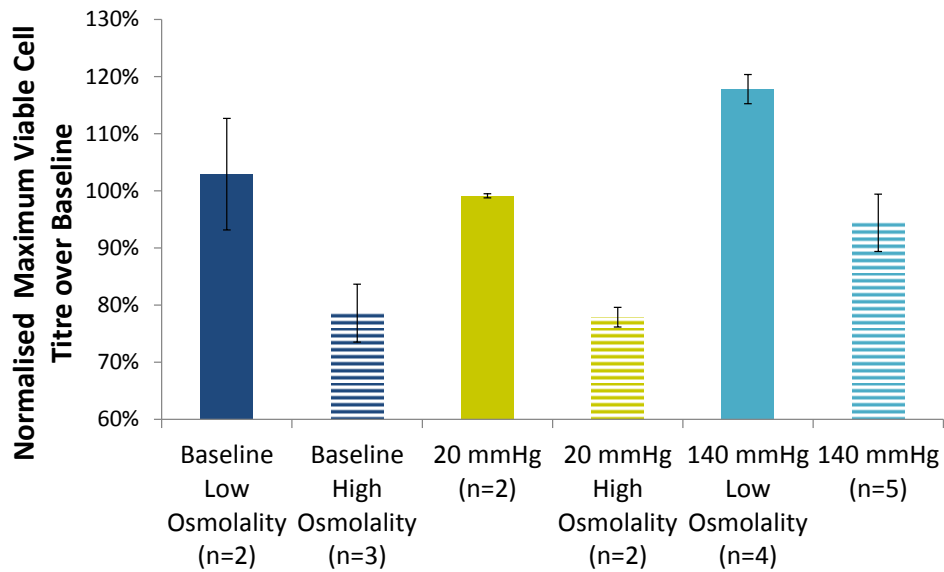
Figure 3.11 - Mean Maximum Viable Cell Titre for pCO$_2$ Control and Osmolality Levels Normalised to the Baseline within Each Batch (Note: Shown with S.E. of the mean solid bars were run at equivalent low osmolality levels, controlled to match the 140 mmHg low osmolality condition and horizontal stripes were ran at equivalent high osmolality levels, controlled to match the 140 mmHg condition. The baseline condition is not shown as the other conditions have been normalised against this condition)

Growth Rate

The effects of osmolality and pCO$_2$ control level on specific growth rate are shown in Figure 3.12. The specific growth rate (μ) is calculated using the equation given in Chapter 2.3.3. The general trend indicates that increased osmolality level results in a decrease in growth rate as shown in Figure 3.12. The highest growth rate was observed at the low pCO$_2$ condition (20 mmHg), which was a condition with a similar osmolality to the baseline.

An equivalent growth rate to the baseline was noted for the baseline low osmolality and 140 mmHg low conditions, with a 4% and 3% reduction, respectively, shown in Figure 3.12. The baseline high osmolality, 20 mmHg high osmolality and 140 mmHg conditions resulted in a reduced growth rate compared to the baseline with a 10%, 12% and 18% decrease, respectively. The highest growth rate was observed in the 20 mmHg condition, with a 14% increase in growth rate compared to the baseline.
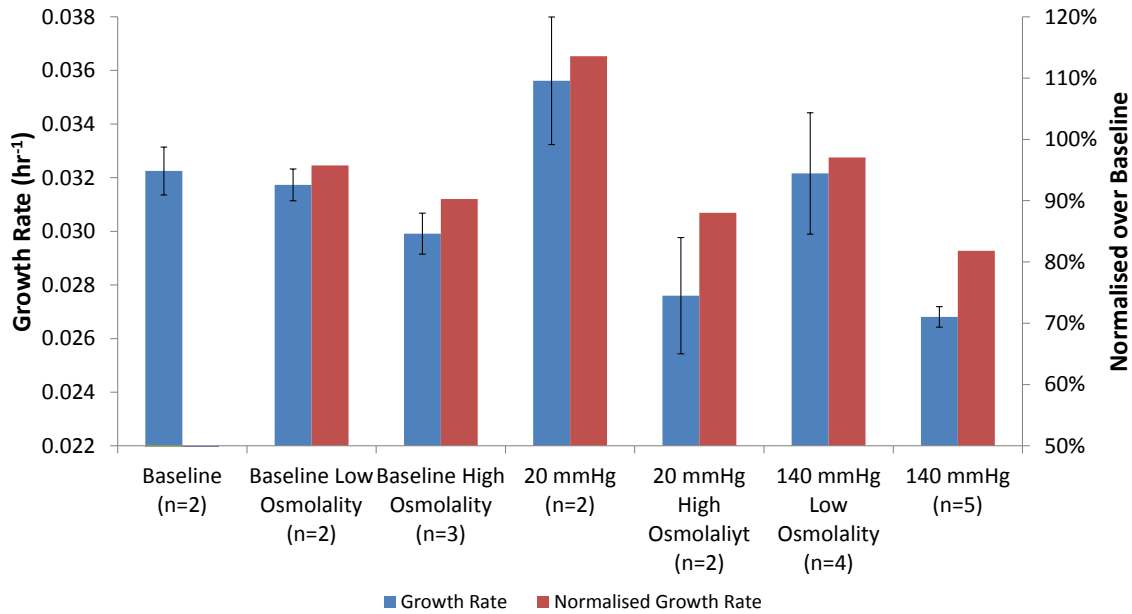
Figure 3.12 - Effect of pCO$_2$ Control and Osmolality Level on the Specific Growth Rate of Viable Cells and the Percentage Normalised against the Baseline (Note: Shown with the S.E.)

3.3.4 Cell Metabolism

Changes to both the osmolality and pCO$_2$ control levels impacted on the cell metabolism and this was demonstrated through a change in the metabolism profiles of ammonium, lactate and key amino acids.

Lactate and Ammonium

The overall trend indicated that elevated osmolality resulted in reduced ammonium levels. Operating at a high osmolality level compared to low osmolality levels, whilst maintaining the pCO$_2$ control level, resulted in lower levels of ammonium production during the stationary phase (Figure 3.13 (b) and Figure 3.14).

Control of pCO$_2$ at 140 mmHg, irrespective of osmolality level, resulted in a change in the ammonium metabolism compared to all other conditions (as was seen in the previous study, Section 2.3.4). Operation at the baseline and 20 mmHg resulted in the re-metabolism of ammonium at the mid-exponential point (approximately 72 to 96 hours), which did not occur in the 140 mmHg condition, shown in Figure 3.13 (b). In contrast, at 96 hours, the ammonium production rate slowed, but re-metabolism did not occur for the 140 mmHg conditions.

For the baseline and baseline low osmolality level conditions, there is a tail off in ammonium production post 156 hours, as observed in the previous study (Section 2.3.4), which coincides with a

reduction in mAb production compared to the other control levels. The reduced ammonium in the baseline and baseline low osmolality conditions are considered to be an indication of a lower TCA cycle activity (as ammonium is a by-product of the TCA cycle).
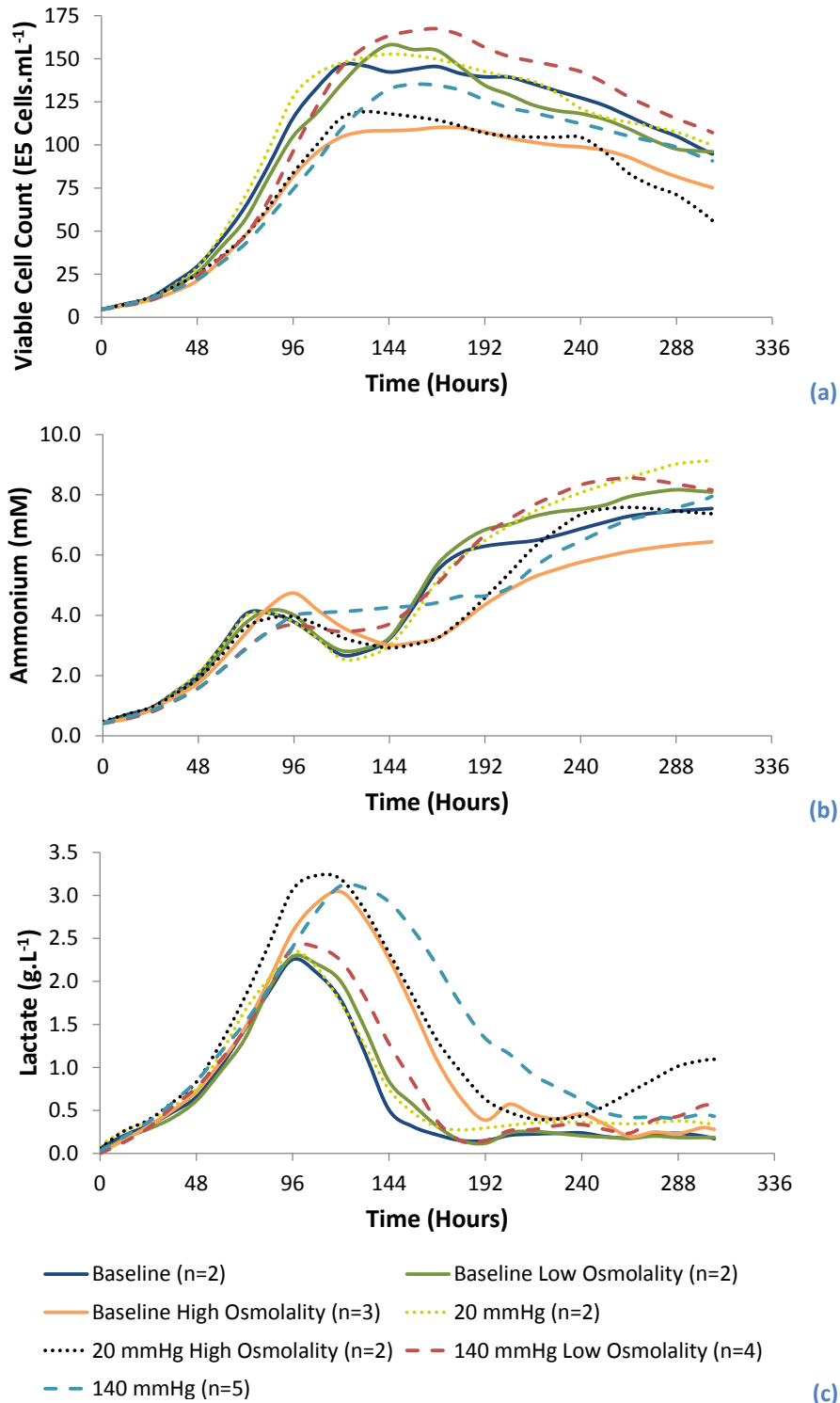
Figure 3.13 - Time Profile of Mean (a) Viable Cell Count (b) Ammonium (c) Lactate for pCO$_2$ Control and Osmolality Levels (Note: Data points have been interpolated to new equal time points by linear interpolation to determine mean values)

The lowest harvest ammonium levels were observed in the baseline high osmolality condition. Conditions with the same pCO_2 control level and increased osmolality resulted in reduced ammonium levels at harvest, shown in Figure 3.14. The opposite trend in the lactate was observed, i.e. increasing lactate levels with higher osmolality, of which the relationship between lactate and ammonium was discussed in detail in Section 2.4.4.
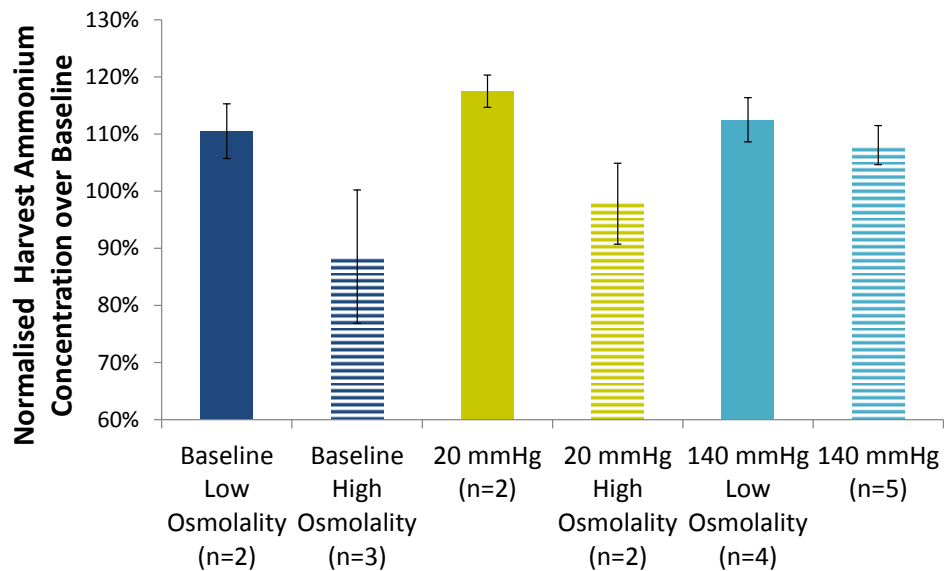
Figure 3.14 - Mean Maximum Ammonium Level for pCO$_2$ Control and Osmolality Levels Normalised to the Baseline within Each Batch (Note: Shown with S.E. of the mean solid bars were run at equivalent low osmola0lity levels, controlled to match the 140 mmHg low osmolality condition and horizontal stripes were ran at equivalent high osmolality levels, controlled to match the 140 mmHg condition)

An increase in the osmolality level resulted in increased peak lactate concentration, with no change observed as a result of a change in pCO_2 control level. Maintaining pCO_2 control level and elevating the osmolality level resulted in a 29% increase in peak lactate concentration for the baseline, 39% at 20 mmHg and 31% at 140 mmHg (Figure 3.13 (c) and Figure 3.15).

The time series lactate trend was unaffected for all control levels, with an exponential increase from the start to mid-exponential growth phase and a switch to consumption from the mid-exponential to the stationary phase. Operation at 140 mmHg resulted in a delay in the switch to re-metabolism of lactate by approximately 12 hours, which coincided with a shift in the depletion of Glx/Asn as observed in the previous study (Section 2.4.4).
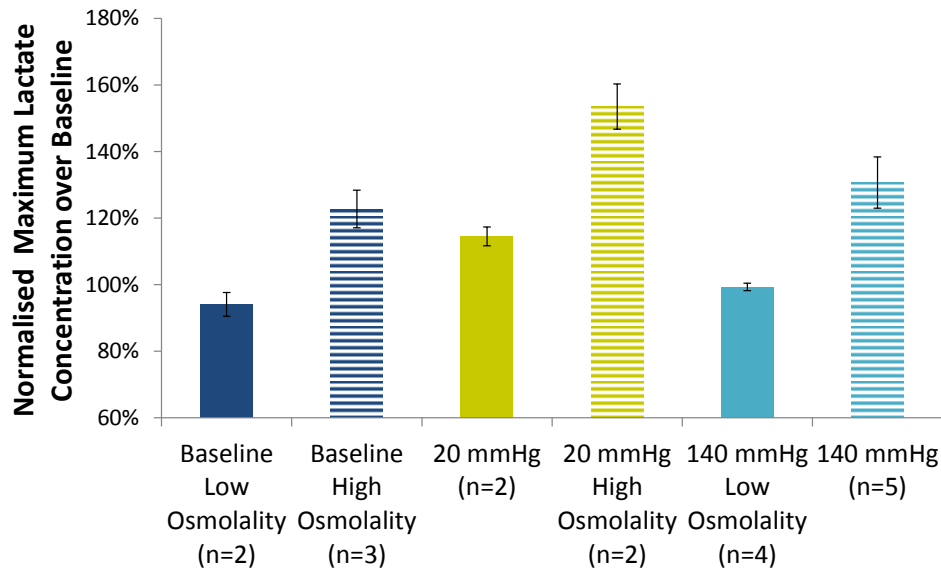
Figure 3.15 - Mean Maximum Lactate Level for pCO$_2$ Control and Osmolality Levels Normalised to the Baseline within Each Batch (Note: Shown with S.E. of the mean solid bars were run at equivalent low osmolality levels, controlled to match the 140 mmHg low osmolality condition and horizontal stripes were ran at equivalent high osmolality levels, controlled to match the 140 mmHg condition)

Key Amino Acids

The key amino acids presented in this section are those which showed a difference relating to pCO$_2$/osmolality control level and are discussed in the literature as having an impact on cell performance.

The consumption of Glx and Asn for all conditions is closely related, as observed in the previous study (Chapter 2), as shown in Figure 3.16. Operation at 140 mmHg resulted in a delay in the depletion of Glx and Asn, by approximately 12 hours, whereas no real difference in the Glx/Asn consumption profiles was observed for all other conditions. No further differences were observed relating to the control of pCO$_2$ and osmolality from the previous study.

(a)

Baseline (n=2) — Baseline Low Osmolality (n=2)
Baseline High Osmolality (n=3) — 20 mmHg (n=2)
20 mmHg High Osmolality (n=2) — 140 mmHg Low Osmolality (n=4)
140 mmHg (n=5)

(b)
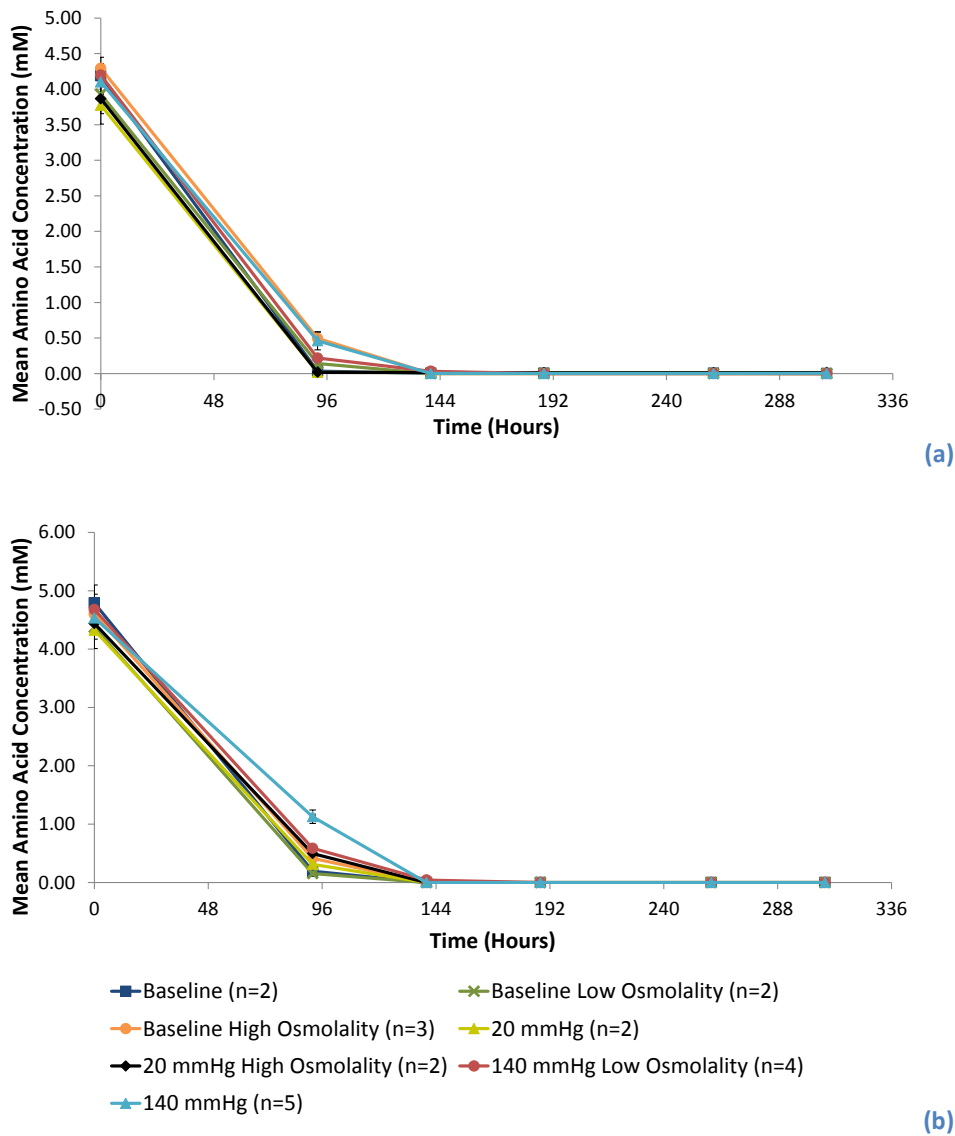
Figure 3.16 - L-alanine-L-glutamine, Glx (a) and Asparagine, Asn (b) Concentration Time Profiles for pCO$_2$ Control and Osmolality Levels (Note: Shown with S.E. of the mean)

The alanine and lactate profiles are closely related (due to the metabolism of glutamine and pyruvate), with an initial production phase from the start of the culture until the mid-exponential point, after which alanine and lactate are consumed. The most significant change to the alanine metabolism occurred for the 140 mmHg and baseline high osmolality conditions. At 140 mmHg, the lowest levels of alanine were observed, as well as a reduction in the consumption rate during the stationary phase. The baseline high osmolality condition had the highest alanine concentration with a reduction in the alanine consumption rate.

The pCO$_2$ control level affected the production rate of glutamate (Figure 3.17 (c)). Glutamate is normally consumed in the TCA cycle and is fed as a substitute for glutamine, however, this cell line showed a production of glutamate. With pCO$_2$ control, the production rate of glutamate was

lowered. Similar production rates occurred in the baseline condition, irrespective of osmolality, and the lowest production rate was observed in the 20 mmHg and 20 mmHg high osmolality conditions.
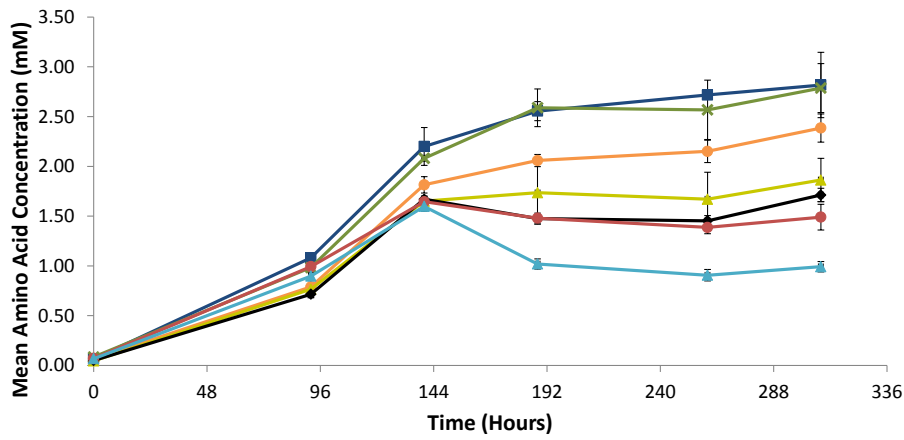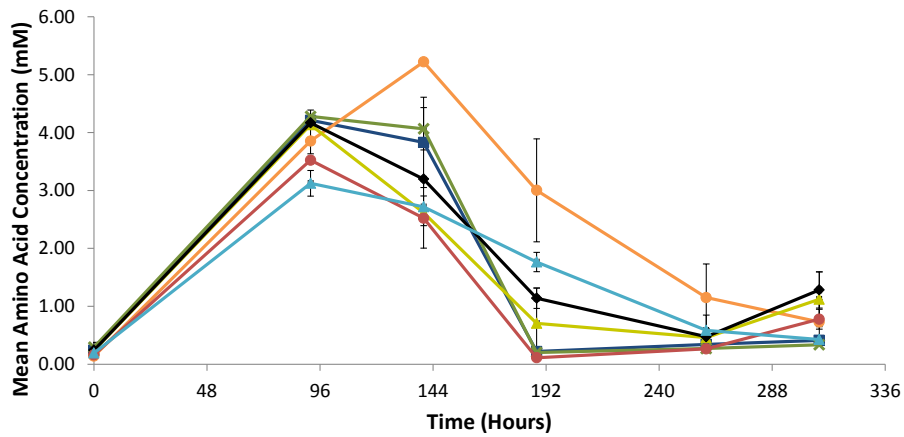
(a)

(b)
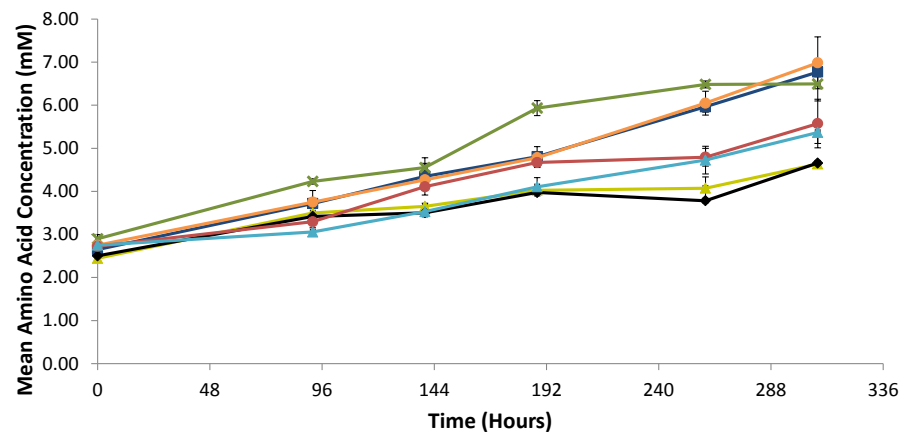
Legend:
- Baseline (n=2)
- Baseline Low Osmolality (n=2)
- Baseline High Osmolality (n=3)
- 20 mmHg (n=2)
- 20 mmHg High Osmolality (n=2)
- 140 mmHg Low Osmolality (n=4)
- 140 mmHg (n=5)

(c)

Figure 3.17 - Glycine (a), Alanine (b) and Glutamate (c) Concentration Time Profiles for pCO$_2$ Control and Osmolality Levels (Note: Shown with S.E. of the mean)

3.4 Discussion

3.4.1 Productivity

Increased pCO_2 control and osmolality level resulted in an increase in mAb titre and cell specific productivity compared to the baseline condition, which replicates a traditional cell culture process where pCO_2 is adjusted to maintain pH. For the reasons given in Section 3.1, it had been considered that the osmolality increase discussed in Chapter 2 would not be great enough to affect the process significantly. However, the results of the study indicated that this was clearly not the case and that the small osmolality change had a much greater effect on this cell line/process than was previously considered.

The results indicate that osmolality and pCO_2 have an effect on the product titre, shown in Figure 3.18. Examining conditions with an increased pCO_2 at a similar osmolality level, resulted in a comparable increase in harvest mAb titre. For example, increasing pCO_2 from the baseline control to 140 mmHg led to a 28% increase while maintaining osmolality at a low level and 29% increase at a high osmolality level (Figure 3.18).
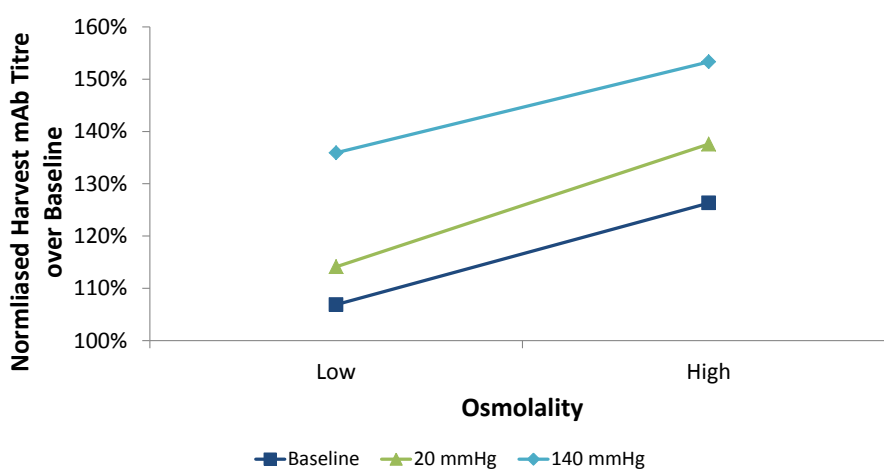
Figure 3.18 - Increase in Normalised Harvest mAb Titre with Osmolality and pCO_2

When the pCO_2 control level was maintained and osmolality level increased, an equivalent increase in mAb harvest titre occurred between the low and high osmolality conditions. For example, maintaining pCO_2 at the baseline condition or at 140 mmHg and increasing osmolality from low to high resulted in a 19% and 20% increase in harvest mAb titre, respectively (Figure 3.18).

The highest mAb titre was achieved for the 140 mmHg condition, with a 28% higher harvest mAb titre than the baseline, respectively, at low and high osmolality levels. The results suggest that the

increase in mAb titre seen with increasing pCO_2 control (from Chapter 2) is due to the increased pCO_2 and osmolality level.

Figure 3.19 shows that the increase in harvest mAb titre, at increased osmolality, is mainly due to an increase in cell specific productivity. Increasing pCO_2 control alone had a less of an effect on the cell specific productivity, when compared to the effect from the osmolality level. As mentioned previously (Section 3.3.1), the 120% increase at 20 mmHg high osmolality is likely to be batch specific, as the duplicate runs were carried out within one batch and the data was normalised against a baseline within that batch (which varied in cell specific productivity with clone stability). However, despite the effect of clone stability on the productivity of the 20 mmHg batch the overall conclusion would not change on repeat batches. The results demonstrated an increase in productivity with elevated osmolality. Operation at a similar passage number to the other studies would likely have led to an increase in cell specific productivity; however, the magnitude is predicted to be smaller.
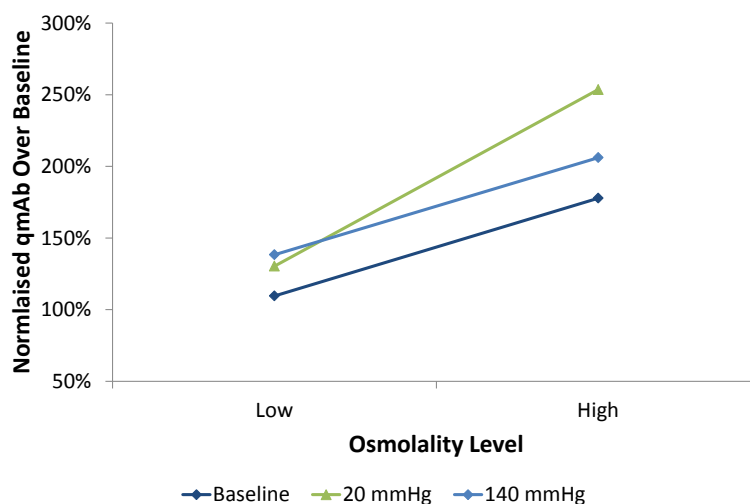
Figure 3.19 - Increase in qmAb with Osmolality and pCO₂ (Note: Values were normalised over the baseline of each condition and the difference determined between the normalised values)

The increase in mAb titre, over the baseline, at low osmolality is due to the combination of a slight increase in cell specific productivity (Figure 3.5) and the increase in cell titre (Figure 3.11), i.e. there are more cells to produce the mAb product. However, caution is required when analysing the cell specific productivity rate, as the calculation is sensitive to variation in cell counts and relies on a model fit. Additionally, the calculation does not take into account changes to cell size (i.e. the cell volume), as lower cell growth and high osmolality were associated with a larger average cell size. Therefore, compared to the cell volume as opposed to cell counts the differences in specific

productivity would be smaller. Additionally, cell activity (i.e. the amount of mAb produced by a cell) may not be directly correlated to the cell count.

Results from the current study, along with those presented in Chapter 2, are in contrast to varied results reported in the literature. A number of the previous studies stated that elevated pCO_2/osmolality level had a negative or limited effect on the productivity of a mammalian cell culture. Previous studies have shown that increased osmolality levels increase cell specific protein productivity, as was observed in this study. However, in a number of literature studies a decrease in cell growth resulted in no increase in overall titre, which was not observed in this study, as discussed in Chapter 2 (Brady, 2009; deZengotita *et al,* 1998; deZengotita *et al,* 2002; Ozturk and Palsson, 1990; Kimura and Miller, 1996; Zhu *et al,* 2005).

In the current study, both osmolality and pCO_2 control level were seen to result in an increase in productivity and product titre. However, as discussed in Chapter 2 (Section 2.4.1), a number of the process parameters were not optimised in previous studies, leading to non-optimal conditions before the additional stresses (of pCO_2/osmolality) were introduced to the system. Also, systems with limited controls and poor process conditions, such as well plates and T-flasks were used (deZengotita *et al,* 1998; Gray *et al,* 1996; Kimura and Miller, 1996; Zhu *et al,* 2005). Additionally, there is limited recent literature of the effect of pCO_2/osmolality on cell culture performance, with the majority of literature published over 15 years ago, in which time there has been advances in cell lines, process monitoring and control, media formulations and a large increase in product titres, which may have resulted in the contrasting results observed in the current study.

As discussed in Chapter 2, the mechanism for the observed increase in productivity at elevated osmolality/pCO_2 is poorly understood. This is mainly due to the contrasting results reported in the literature and the difficulty in analysing cell culture data due to the complex behaviour inherent to the cell metabolism. Theories proposed include enhanced nutrient transport into the cell, increased mass per cell, cell cycle arrest in a more efficient stage, increased transcription/translation rates and the alteration of the pH_i, as discussed in Section 2.4 (Brady, 2009; deZengotita *et al,* 1998; Eisenkraetzer *et al*, 2013; Lin *et al*, 1999; Silva *et al*, 2013). For an enhanced understanding of the mechanism through which pCO_2/osmolality affects the cell culture process, analysis of the pH_i and metabolic flux analysis is required, in order to monitor the cell metabolism and the effect of pCO_2 on the pH_i and cell metabolism. Methods for the analysis of internal metabolism and measurement have been presented by a number of prevous studies, however, these methods are offline, costly, time consuming and require a number of modelling assumptions (Dean and Reddy, 2013; Duarte *et al*, 2014; deZengotita *et al*, 2002; Templeton *et al,* 2013).

In summary, in addition to the results presented in Chapter 2, the results reported in this chapter demonstrate that both pCO_2 and osmolality affect the productivity of a CHO cell line process. Increasing the pCO_2 control and osmolality level separately resulted in increased product titre over a traditional cell culture process (the baseline condition). These results are in contrast to a number of previous studies that reported varying effects of pCO_2/osmolality on product titre. The differences between the current and previous studies are considered to be likely due to process differences, including vessel and media type, leading to interacting effects from other parameters, as discussed previously.

As the osmolality and pCO_2 are closely related, the assessment of the independent effect of each parameter on cell culture performance is not possible in a traditional batch culture. Operation under a perfusion culture, which is a semi-continuous, pseudo-steady state operation would allow for a certain degree of decoupling of pCO_2 and osmolality level. Perfusion cultures operate by constantly replenishing the culture media, resulting in the removal of waste products and replenishment of nutrients, therefore, allowing for the reduction in the dynamics of the system. The reduced dynamics allows for a more constant culture pH, therefore, through design of the culture media a constant pCO_2 level independent of osmolality could be assessed.

The differing effect of pCO_2 and osmolality level on cell culture processes, as reported in the literature, highlights the need for the consideration for pCO_2/osmolality monitoring and control in cell culture processes. The contrasting results published in the literature suggest that process and cell line specifics are significant in terms of the effect of pCO_2/osmolality on cell culture processes. Therefore, the consideration of pCO_2/osmolality during small scale, scale-up and QbD studies, is essential to determine if they are critical process parameters. For this process and cell line, the pCO_2 control and the osmolality level have been shown to be critical process parameters.

3.4.2 Product Quality

In the previous study discussed in Chapter 2, it was observed that control of pCO_2 at low to high levels (20 to 100 mmHg) resulted in a significant decrease in the product quality attributes, compared to the baseline and elevated pCO_2 (140mmHg) conditions. In the current study similar results were observed with reduced product quality attributes at 20 mmHg and equivalent product quality observed at 140 mmHg compared to the baseline.

The results indicate that pCO_2 has a more significant effect on product quality for the range tested. Operation at low pCO_2 control level (20 mmHg), regardless of osmolality level, resulted in a decrease in the product quality attributes compared to the baseline and 140 mmHg conditions, with the

highest target mAb observed for the 140 mmHg condition. Comparing runs of the same pCO_2 control level where osmolality was modified (e.g. 20 mmHg versus 20 mmHg high osmolality) led to minimal or no change in the product quality profile.

Carbon dioxide concentration affects the charge variants through a significant increase in the basic variant composition and a slight decrease in the acidic variant composition, compared to the baseline condition. Alongside the previous study (Chapter 2), the results indicate that increasing pCO_2 control from 20 to 140 mmHg leads to a decrease in the basic variants composition. The formation of acidic and basic variants can occur due to a number of degradation pathways as shown in Table 3.4; however, full mAb profiling is required to determine the cause of the variant formation. This would aid in determining the cause of the change in composition of the mAb product, i.e. the degradation pathway.

Major Chemical Degradation Pathways	Effect	Species
Sialylation	COOH addition	Acidic
Deamidation	COOH formation	Acidic
C-terminal lysine cleavage	Loss of NH_2	Acidic
Adduct formation	COOH formation of loss of NH_2	Acidic
Succinimide formation	Loss of COOH	Basic
Methionine, cysteine, lysine, histidine, tryptophan oxidation	Conformational change	Basic
Disulfide-mediated	Conformational change	Basic
Asialylation (terminal Galactose)	Loss of COOH	Basic
C-terminal lysine and glycine amidation	NH_2 formation of loss of COOH	Basic

Table 3.4 - Major Chemical Degradation Pathways that Lead to Formation of Acidic/Basic Charge Variants (Khwali et al, 2010)

Osmolality had limited impact on product quality for the range tested, when controlling pCO_2, with little change in the peak target mAb (144 hours) when osmolality was modified at 20 and 140 mmHg. Throughout the culture a greater increase in the acidic and basic variants was observed for the 140 mmHg low osmolality conditions than for the 140 mmHg conditions, resulting in up to a 6% difference in the target mAb. The lower target mAb is possibly due to the increased levels of ammonium production at the 140 mmHg low osmolality level compared to 140 mmHg. Ammonium levels have been reported to impact product quality; however, the effect of ammonium level on this process requires further investigation (Schneider *et al*, 1996; Sengupta *et al*, 2011).

There is limited literature on the effect on pCO_2 and osmolality on product quality. The current study results differ to those reported in the literature which indicated that both pCO_2 and osmolality have little effect on the product quality attributes (Kimura and Miller, 1997; Schmelzer and Miller, 2002). The difference in the results is likely due to the differences as discussed in Chapter 2 (Section 2.4).

As discussed in the previous chapter (Chapter 2), there are a number of potential causes for the effect of pCO_2/osmolality on product quality. Carbon dioxide affects the pH_i, as it is readily diffused into the cell and dissociates to H^+ and HCO_3^-, therefore affecting the pH_i. Therefore, it is likely that the change in pH_i, caused by pCO_2, will affect the cell metabolism and hence the product quality. However, measurement of pH_i during the process, alongside metabolic flux analysis, is required to confirm this theory. Also, pCO_2 potentially plays a role in increasing the efficiency of the cell metabolism, for example, through the increased flux of malate into the TCA cycle, which may increase the activity of the TCA cycle as reported by Eisenkraetzer *et al* (2013).

It is unlikely that the main cause of the differences in product quality is due to process intermediates such as lactate and ammonia. For the baseline high/low osmolality conditions there is a significant difference in the lactate and ammonia concentrations but very little difference in the product quality attributes. It is, however, possible that these variables play an interacting effect on the product quality with a number of other factors, but further investigation is required.

In summary, the results indicate that the pCO_2 control level has the greatest impact on product quality, with the main effect being an increase in basic variants and a slight decrease in acidic variants, with decreasing pCO_2 control level. Osmolality appears to have minimal impact on product quality for the range investigated, with a slight decrease in acidic and small increase in basic variants with increasing osmolality.

It may be possible to determine if the effect on product quality is due to degradation of the mAb in the cell culture environment or as a consequence of the "health" of the cells themselves during the cell culture. To investigate this, an experiment could be performed using purified mAb. The purified mAb could be incubated in spent media, under the same conditions as the cell culture, to determine to what extent the degradation is due to the environmental conditions. This was outside of the scope of the current investigation.

3.4.3 Cell Growth

A large amount of variation was observed in the cell growth data, in terms of maximum cell titre and growth rate (Figure 3.11 and Figure 3.12). Variations in cell titre observed are likely due to clone line stability and variations in STR-to-STR performance; however, the cause of the variations is not fully understood. Therefore, only macro differences in the cell growth could be analysed from the study, as variations between control conditions may have been masked by the variation in the process.

The general trend indicates that the higher osmolality level results in reduced cell growth, which is in agreement with the literature (Gray *et al*, 1996; Kimura and Miller, 1998; Zhu *et al,* 2005). The 140

mmHg low osmolality condition had the highest viable cell density, which is a result which has not been observed in the literature, with studies suggesting the elevated pCO_2 reduces cell titre (Gray *et al,* 1996; deZongotita *et al*, 1998; Kimura and Miller, 1996). The results from the current study support the findings reported in the literature and the study described in Chapter 2 that elevated osmolality results in lower cell growth, with pCO_2 control level playing a lesser role.

The changes to cell growth with pCO_2/osmolality did not have a negative impact on the cell culture, in terms of cell viability and productivity, excluding the 20 mmHg high osmolality condition which was harvested 12 hours earlier than the other conditions. This may indicate that at low pCO_2 high osmolality the cells are more sensitive to osmolality levels.

The results of the current study differ from a number of previous studies where decreased cell titre was reported to have negatively impacted on the final mAb titre (Ozturk and Palsson, 1990; deZengotita *et al*, 1998). The differences in results may be due to cell line and process specifics; however, more likely due to the differences as discussed in Chapter 2, concerning the vessel type, interacting process parameters and advancements in cell lines/processes since the publications.

The cause of the inhibitory effect of elevated pCO_2/osmolality on cell growth is not fully understood. Possible theories on the effects of pCO_2/osmolality include the changes to the pH_i, which affects pH dependant processes within the cell, and the direct effect of elevated pCO_2/osmolality levels on the cell metabolism (Gray *et al,* 1996; deZongotita *et al*, 1998; Kimura and Miller, 1996). The osmolality of the culture will affect the nutrient/ion composition and osmolality inside the cell, which, in turn, can affect the cell metabolism. Therefore, metabolic flux analysis and analysis of pH_i under varied levels osmolality and pCO_2 are required to aid in determining the effect.

Reduced cell growth and increased product titre is an advantage for downstream processing (DSP). The first step of DSP is the removal of cells. With the push towards disposable systems, alternatives are required for the centrifugation step for single-use facilities. One option is the use of depth filtration as a cell removal step. For depth filtration the number of cells and particularly the number of lysed cells can present issues. Therefore, a reduction in the number of cells and accordingly the number of lysed cells will aid in DSP.

3.4.4 Cell Metabolism

The general trend reported in this study agrees with previous investigations into the effect of elevated pCO_2/osmolality on lactate and ammonium, with an increase observed at elevated pCO_2/osmolality levels (Ozturk and Pallson, 1990; Schmelzer and Miller, 2002; Zhu *et al*, 2005).

Lactate

The results indicate that pCO$_2$ control alone has no effect on the lactate metabolism and that the increase in lactate concentrations observed with higher pCO$_2$ control (in Chapter 2) is due to the associated osmolality increase. Conditions with similar osmolality levels gave rise to a similar lactate metabolism, irrespective of pCO$_2$ level, with increased peak concentrations at high osmolality.

A shift in the re-metabolism of lactate was seen, for the 140 mmHg conditions, by approximately 12 hours, which did not occur in the other conditions. This is likely linked to the 12 hour delay in the depletion of Glx/Asn for this condition, which was discussed in detail in Section 2.4.4. This further supports the theory that Glx and Asn play a role in the switch in lactate and cell growth as both have been shown to impact lactate and cell growth metabolism (Dean and Reddy, 2013; Durate 2014).

The pCO$_2$ and osmolality levels had little impact on the glucose and glutamine consumption, which are the main sources of lactate production. Lactate has previously been reported as being transported out of the cell due to a decrease in pH$_i$, which may be the cause of the increased concentration observed at elevated osmolality (Eisenkraetzer *et al,* 2013; Silva *et al,* 2013).

Another possible cause of the increased lactate is a combination of the glutamine and glucose metabolism and reduced cell growth, as discussed in Section 2.4.4. To aid in determining the cause of the increase in lactate concentration further understanding of the internal cell metabolism is required, through metabolic flux analysis.

Ammonium

Controlling pCO$_2$ with a low osmolality level led to higher levels of ammonium production throughout the course of the culture compared to pCO$_2$ control at high osmolality. This is likely to be linked to the elevated lactate levels and reduced alanine consumption rate at the higher osmolality level. As discussed in Chapter 2, the consumption of lactate substitutes the catabolism of amino acids which would result in the production of ammonium. For example, alanine consumption is the primary source of ammonium accumulation once lactate has been depleted and increased levels of lactate limit the consumption of alanine (Dean and Reddy, 2013; Li *et al*, 2012; Zagari *et al*, 2013).

The likely cause of the lower ammonium in the baseline conditions compared to the 140 mmHg low osmolality and 20 mmHg conditions is due to decreased TCA activity, shown in terms of reduced production titre and reduced amino acid consumption in the stationary phase.

When controlling pCO$_2$ at 140 mmHg, irrespective of osmolality, ammonium was not re-metabolised between the mid-exponential and stationary phases. This indicates a potentially significant change in

the cell metabolism as a result of elevated pCO_2. Metabolic flux analysis is required in order to examine the changes to the entire metabolism as a result of pCO_2/osmolality.

3.5 Conclusions and Further Work

In the previous study (Chapter 2) the pCO_2 control level was shown to affect the cell culture process, in terms of product titre, product quality and cell metabolism/growth. An associated osmolality increase was observed with increasing pCO_2 control. Therefore, in this study, to determine the role of the osmolality increase with increasing pCO_2 control, the osmolality was adjusted to low and high levels at different pCO_2 control levels (baseline, 20 and 140 mmHg).

The study results indicated that both osmolality and pCO_2 level independently increased product titre, with a 28% to 29% increase as a result of increased pCO_2 from the baseline to 140 mmHg. A 19% to 20% increase was observed when osmolality was increased from low to high at the baseline and 140 mmHg conditions, respectively. Results also indicated that increasing osmolality led to a significant increase in the cell specific productivity over the baseline. This was due to combination of increased product titre at reduced cell growth.

The results showed that pCO_2 had a more significant impact on product quality, with osmolality playing little or no role. The results discussed in Chapter 2 indicated that pCO_2 affected the charge variants composition, with a reduction in the target mAb at low to high levels (20 to 100 mmHg) and equivalent target mAb at elevated pCO_2 (140 mmHg), compared to the baseline. The results from the current study indicated that the decrease in target mAb observed at low pCO_2 levels was due to pCO_2 level. Osmolality had a less significant impact on charge variants than pCO_2 control level. A similar trend in the charge variants was also observed in end glycan analysis (Figure 3.9); however, only one batch of data was conducted (due to cost and time constraints).

The results from the current study disagreed with a number of literature sources, most likely due to process, process control and cell line differences and also advancements in processing, as the majority of the studies were conducted over 15 years ago. The reported differences highlight the need to consider pCO_2 control and osmolality level during small scale, QbD and process development studies, as the effect may be dependent on process conditions and controls and other interacting effects.

For future work, the design space for osmolality and pCO_2 is required, including the examination of an increased range for the pCO_2 and osmolality, i.e. identification of the failure points. However, for pCO_2 values above 140 mmHg, improved inline and at-line analytics are required, as most

measurement devices are designed to test pCO_2 in the physiological level (about 20 to 70 mmHg). The osmolality range tested was designated by the osmolality level of the 140 mmHg condition and the 140 mmHg condition with NaCl removed.

To aid in determining the cause of the effect due to pCO_2 control and osmolality level, metabolic flux analysis and measurement of pH_i are required. These techniques would aid in determining the effect of pCO_2/osmolality on the (internal/external) cell metabolism, to examine which pathways are affected.

The scale-up of the process into larger vessels is required to determine the effect of pCO_2 on scale-up. The effect of pCO_2 control on other processes, such as differing cell lines, media and reactor types (such as rocker type reactors), is required to determine if the observed results in this study are not due to the process and cell line specifics.

Ideally, the effect of pCO_2 and osmolality should be examined independently and under pseudo-steady state conditions. However, under batch conditions steady state is not achievable as batch cell cultures are dynamic processes. A potential solution is to operate a perfusion cell culture, which is pseudo-steady state. As the culture media in a perfusion process can be removed and replenished and the media can be designed to provide the same osmolality level at different pCO_2 levels (and vice versa), which would allow for step change tests of different pCO_2 and osmolality levels. However, for this process to be valid it would need to be demonstrated that a step change in pCO_2 and osmolality would not affect the ability of the system to return to the pseudo-steady state, i.e. the process can be returned to the same conditions after the step test without effecting the process. In this way, the response directly to pCO_2 or osmolality could be observed.

4 Effect of Carbon Dioxide Control Level on a Mammalian Cell Culture Process under Fed-Batch Mode

Carbon dioxide control was studied under fed-batch mode to assess if the effect of pCO_2 control on a fed-batch process was similar to the effects observed on a batch process as outlined in Chapters 2 and 3. Additionally, a significant number of industrial cell cultures are carried out in fed-batch mode and hence, it is important to understand the effect of pCO_2 in an industrially relevant process. In this context 'fed-batch' refers to the addition of a defined concentrated amino acid feed, fed at specified points in the culture.

The design and optimisation of a fed-batch process is complex and time consuming. Due to time constraints, for this study the fed-batch process was based on the manufacturer's instructions and the amino acid profile from the batch process. Feed additions were designed to prevent the depletion of amino acids before the final feed and entry into the stationary phase.

As the purpose of the study was to determine if the results obtained in the batch study still held relevant under fed-batch conditions, other work (the osmolality study presented in Chapter 3) was prioritised over a fed-batch optimisation, as the optimisation of fed-batch conditions would have little effect on the desired findings of the study. Fed-batch optimisation, including the modification of the base medium formulation, is a long and complex process. Additionally, the medium used in the study was a commercially available medium, making changes to the base formulation difficult as manufactures do not divulge medium information.

Inherently with a fed-batch process the osmolality will increase, as a concentrated feed is added to the process. As a negative control action for osmolality (i.e. removal of solutes) cannot be achieved in fed-batch operation, the control of osmolality is not possible. There is also an added complication of interacting effects within cell culture medium on osmolality level, for example an increase in osmolality leads to an increase in lactate, which subsequently elevates osmolality affecting lactate and osmolality in a cyclic manner. Therefore, the separation of fed-batch and osmolality as factors is not practically possible. Therefore, to simplify the study osmolality was not controlled, but grouped as a factor with the fed-batch. As stated previously, the control of osmolality would not affect the desired outcome of the study, which was to determine if pCO_2 level affected a fed-batch process in a similar manner to observed in Chapter 3.

4.1   Materials and Methods

4.1.1 Cell Line and Medium

A description of the cell line and medium is given in Chapter 2, Section 2.2.1. The feed medium for the fed-batch process was a commercially available type, CD EfficientFeed™ C AGT™ (Life Technologies).

4.1.2 Bioreactor Cell Culture Runs

Details of the bioreactor cell culture runs are provided in Chapter 2, Section 2.2.2. The feeding strategy involved manually feeding a total of 30% of the final volume in 5 equal shots at 24 hour periods from the initial feed. The first feed was undertaken at the start of the mid-exponential phase of viable cell growth. For fed-batch runs, the starting glucose level was maintained at 5 ± 2 g.L^{-1}. At 260 hours all cultures were taken to 10 g.L^{-1} glucose level to prevent glucose depletion over the weekend. The baseline batch condition was operated under the same conditions as the previous batch baseline cultures (Chapters 2 and 3).

4.1.3 pCO$_2$ Control Levels and Feed Strategy

For this study only the elevated pCO$_2$ (140 mmHg) and baseline conditions were examined, to determine if the results observed in Chapters 2 and 3 apply under fed-batch conditions. This was due to the most significant change in cell metabolism, in terms of improved productivity, with maintained product quality attributes, observed at the 140 mmHg conditions compared to the baseline. Details for the bioreactor cell culture runs are given in Chapter 2, Section 2.2.2. For each condition duplicate runs were conducted. The STR test conditions for the study are shown in Table 4.1.

pCO$_2$ Control (mmHg)	Condition	Runs
Baseline	Batch	2
Baseline	Fed-Batch	2
140	Fed-Batch	2

Table 4.1 - STR Test Conditions for pCO$_2$ Fed-batch Study

4.1.4 At-line Analytics

Details for the at-line analytics are given in Chapter 2, Section 2.2.4.

4.1.5 Offline Analytics

Details for the offline analytics are given in Chapter 2, Section 2.2.5

4.2   Results

4.2.1 Antibody Production

Antibody Titre

Figure 4.1 shows the time series plot for mAb titre for the batch and fed-batch conditions. Figure 4.1 illustrates that there was little difference in the mAb production between the fed-batch conditions, irrespective of pCO_2 control level. The fed-batch conditions reached the harvest cell titre, 70-50% cell viability, at approximately 260 hours, whereas the batch condition reached the harvest density at 332 hours. The fed-batch cultures were continued until the harvest point of the baseline was reached, at which point the cell viability was less than 30% (Figure 4.6).

At 260 hours (the harvest point for the fed-batch conditions) the fed-batch process resulted in a 126% to 135% increase in mAb titre (Figure 4.2) over the baseline, with no significant difference between the 140 mmHg and baseline fed-batch conditions. At 332 hours (the harvest point for the batch process), a 120% to 134% increase was observed. Again, there was no significant difference between the fed-batch conditions, with 6% difference between the mAb titres for the fed-batch conditions (0.66 g.L^{-1} and 0.70 g.L^{-1} for the 140 mmHg and baseline, respectively). The 14% difference is accentuated in Figure 4.2, due to normalisation against the baseline batch condition (which was off a low value). The tail off in mAb production for the 140 mmHg fed-batch condition is likely to be due to the sharp decrease in cell titre and culture viability (shown in Figure 4.6).
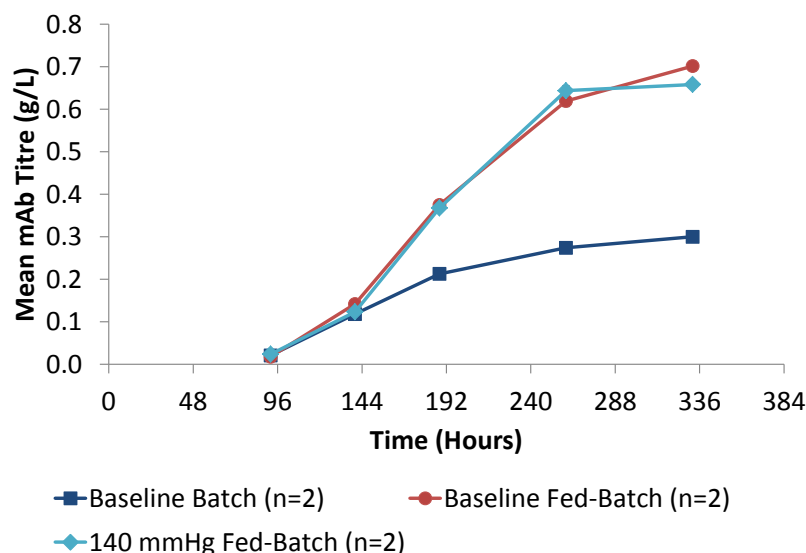
Figure 4.1 - Mean mAb Titre for Batch and Fed-Batch Conditions (Note: Shown with S.E. of the mean)
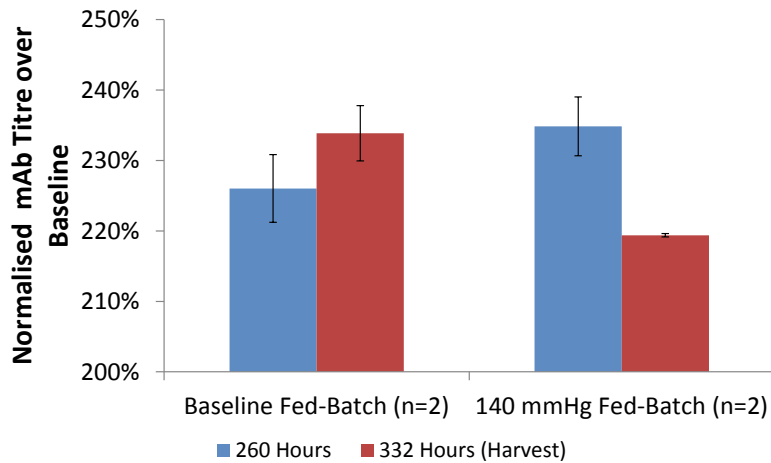
Figure 4.2 - Harvest mAb Titre Normalised over the Baseline Condition within Each Batch (Note: Shown with S.E. of the mean calculated using the normalised values)

Cell Specific Productivity

Figure 4.3 indicates a significant increase in the cell specific antibody production rate under fed-batch conditions. A 380% to 450% increase was observed for the 140 mmHg fed-batch condition and 150% to 200% for the baseline fed-batch, compared to the baseline batch condition. The cell specific productivity for the 140 mmHg fed-batch condition was almost twice that for the baseline fed-batch condition.
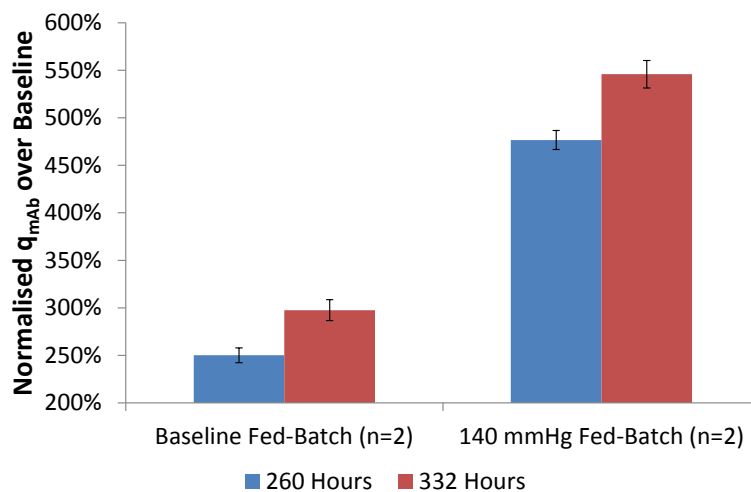
Figure 4.3 - Cell Specific Antibody Production Rate Normalised to Baseline Cell Specific Productivity (Note: Shown with S.E. of the mean calculated using normalised values)

Charge Variants

The 140 mmHg fed-batch condition resulted in an improved target mAb composition compared to the baseline batch and fed-batch conditions as illustrated in Figure 4.4 (a). For the 140 mmHg fed-batch, a 22% increase occurred in the target mAb composition compared to the baseline conditions (batch and fed-batch) at 192 hours. Post 192 hours, the difference between the target mAb composition for the baseline and 140 mmHg conditions decreased, which was due to the dramatic decline of the cell culture viability (see Figure 4.6). The decline in cell culture viability for the fed-batch conditions was due to operating an non-optimised fed-batch process. As observed in the previous study (Chapters 2 and 3), the decline in target mAb composition is correlated with the decline in culture viability.

The improved target mAb composition at the 140mmHg condition is due to a combination of reduced levels of both acidic and basic charged variants. Figure 4.4 (b) illustrates that the fed-batch conditions (baseline and 140 mmHg) had identical acidic profiles, which were both lower compared to the batch condition from 144 to 260 hours. In contrast, the basic variants for the 140 mmHg fed-batch and baseline batch conditions are similar, with an increase in basic variants for the baseline fed-batch condition, shown in Figure 4.4 (c). The results suggest that pCO_2 control under fed-batch conditions has a greater effect on the basic variants composition.

As explained in the previous Chapters 2 and 3, the main cause of the decrease in target mAb composition is due to a time dependant increase in acidic variants. At 144 hours the acidic variant composition is very similar for all conditions. At this point in the culture there is little difference in the mAb titre as this is the start of the stationary phase in which the bulk of the mAb is produced, suggesting either that the addition of the feed or the increased mAb concentration leads to decreased acidic variants.
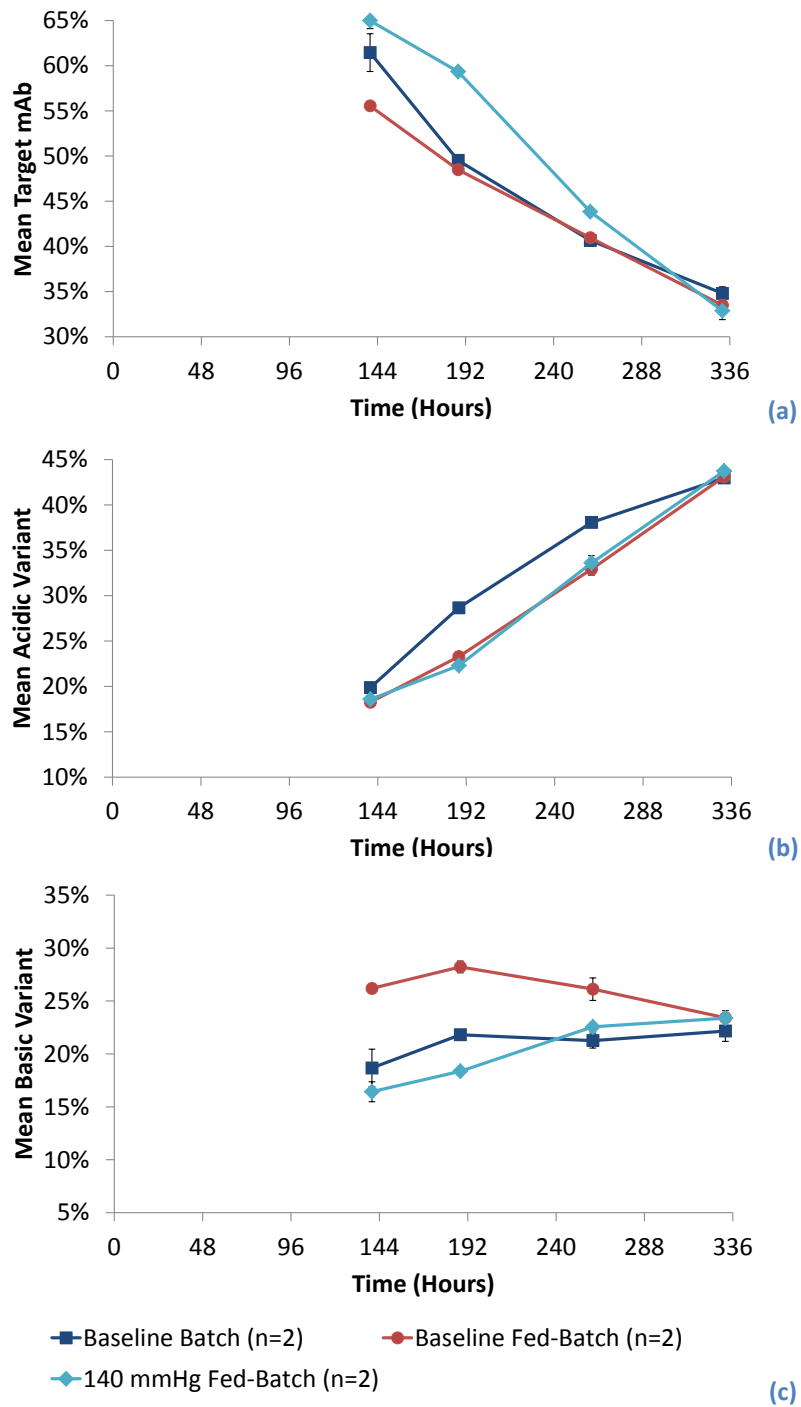
Figure 4.4 - Target mAb (a) Acidic (b) and Basic Variant (c) Composition for Batch and Fed-batch Conditions

4.2.3 Cell Growth

Viable Cell Growth Profile and Maximum Cell Titre

Figure 4.5 and Figure 4.6 show that the fed-batch baseline process led to an increased maximum viable cell concentration over the baseline, with a 27% increase. The 140 mmHg fed-batch condition resulted in a slight decrease in the maximum cell titre, 13%, compared to the baseline batch condition. However, the maximum cell titre for the 140 mmHg fed-batch condition resulted in a 10% increase compared to the to the maximum cell titre obtained for the 140 mmHg batch conditions in the previous study, discussed in Chapter 2.
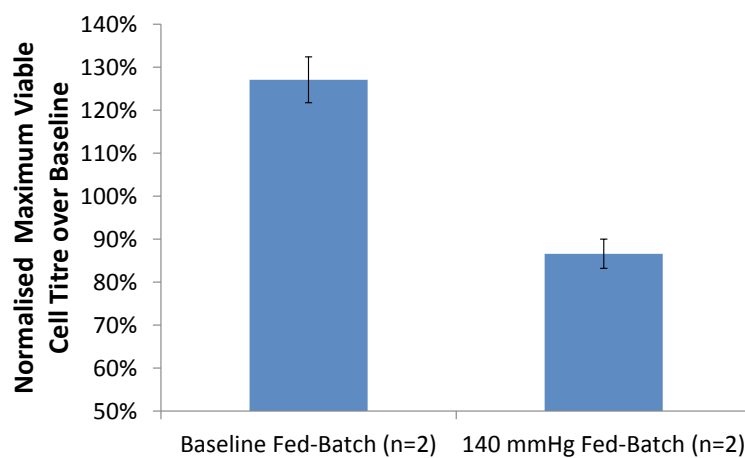
Figure 4.5 - Cell Viability for Batch and Fed-batch Conditions Normalised to Baseline (Note: Shown with S.E. of the mean)

Figure 4.6 indicates that despite the increased cell titre under fed-batch conditions, the time at which the cells entered the stationary phase was unaffected. This suggests that one or a number of specific growth-related amino acids were depleted at the same rate, due to an increased consumption rate with increased cell concentration in the fed-batch process (i.e. depleted at the same time point as the batch culture). Alternatively, it may be due to a growth related amino acid not being present or not being at a high enough concentration in the feed, i.e. optimisation of the fed-batch process is required.
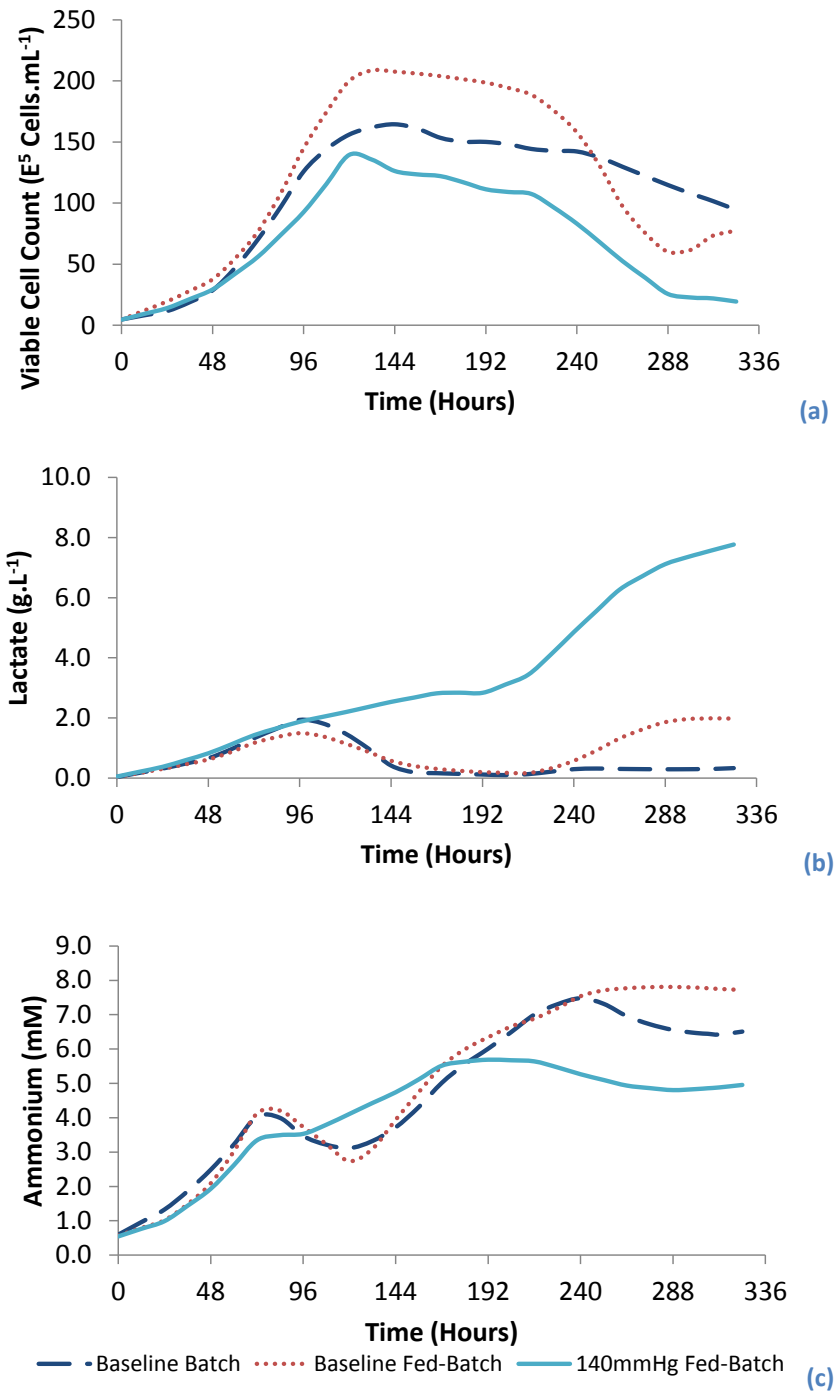
Figure 4.6 - Viable Cell Growth (a), Lactate (b) and Ammonium Profile (c) for Batch and Fed-batch Conditions (n=2)

4.2.4 Cell Metabolism

Lactate and Ammonia

Figure 4.6 (b) indicates the lactate metabolism for the fed-batch and batch conditions over the course of the cell culture. There is minimal difference in the lactate metabolism during the mid-exponential growth phase between the batch and fed-batch conditions, 0 to 96 hours, shown in Figure 4.7, due to the first feed not occurring until 72 hours.
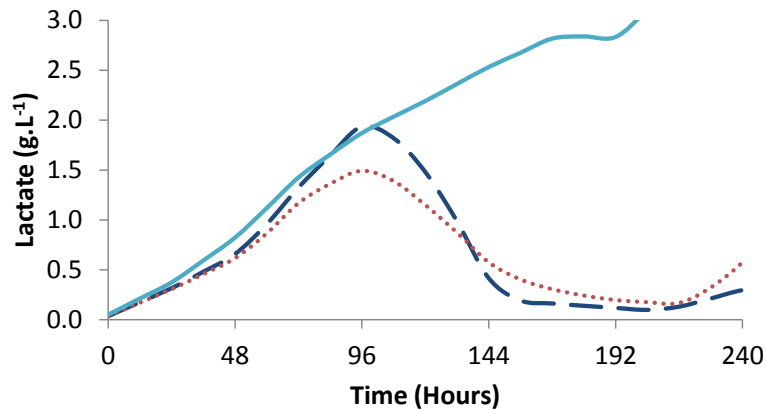
Figure 4.7 - Lactate Profile During the Exponential Growth Phase (n=2)

As shown in Chapter 3, this is likely due to the effect of osmolality on the lactate metabolism. As glucose was not pre-loaded (i.e. taken to 15 g.L^{-1} at the start of culture) for the fed-batch conditions, the 140 mmHg fed-batch osmolality was comparable to that of the batch baseline, resulting in similar lactate profiles. The baseline fed-batch condition had approximately 40-50 mOsmo.kg^{-1} lower osmolality (Figure 4.8), and a slightly lower lactate concentration of 25%, shown in Figure 4.7. This supports the conclusions arrived in Chapters 2 and 3 that increased osmolality results in increased lactate concentrations.
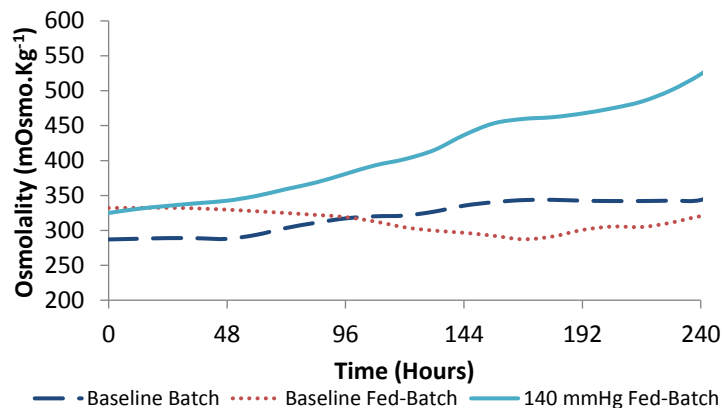
Figure 4.8 - Osmolality Time Profile for Batch and Fed-batch Conditions

For the 140 mmHg fed-batch culture, lactate was not re-metabolised. This is likely due to amino acids not being depleted and therefore, not requiring lactate as an energy source. When the culture went into a dramatic decline in viability the production rate of lactate increased, resulting in high levels of lactate at the end of the culture. From experimental observations an increase in lactate for this process indicates undesirable process conditions. This undesirable condition is considered to be due to the increase in osmolality (Figure 4.8), which is the likely cause of the increased cell death seen for the 140 mmHg fed-batch conditions. An increase in lactate concentration can also be seen for the baseline fed-batch conditions post 240 hours, when the cell titre began to dramatically drop, which is shown in Figure 4.6.

Unlike the lactate metabolism, the fed-batch process did not have a significant effect on the ammonium metabolism. Figure 4.6 (c) demonstrates that the batch and fed-batch baseline conditions had similar ammonium profiles. The 140mmHg fed-batch condition had a similar trend as was seen in previous batches for the batch 140mmHg condition (Chapters 2 and 3), i.e. no re-metabolism of ammonium occurred (prior to the harvest point). The ammonium levels for all conditions are very similar, until a deviation at 192 hours for the 140 mmHg fed-batch and 240 hours for the baseline fed-batch, which is due to the decline in cell viability below the harvest value.
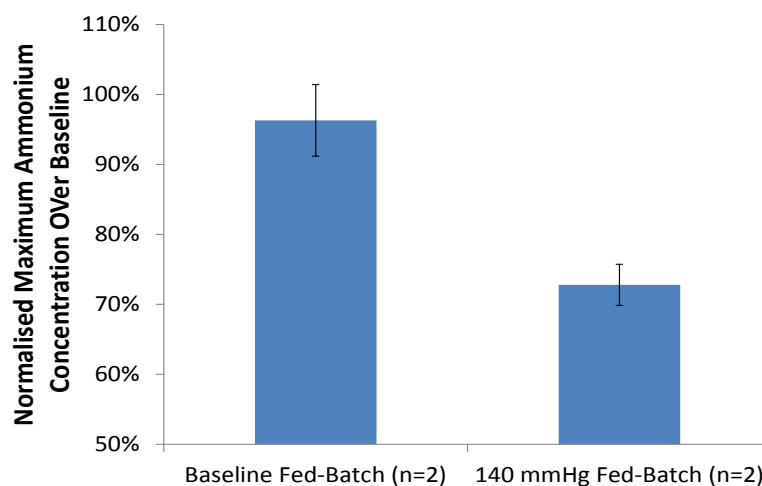
Figure 4.9 - Mean Maximum Ammonium Concentration Normalised to the Baseline (Note: Shown with S.E. of the mean)

Key Amino Acids

Figure 4.10 illustrates that ultraglutamine (Glx) and asparagine (Asn) were depleted at the same point in the batch and fed-batch conditions. This is due to the increased consumption of Asn/Glx in

the fed-batch conditions as a result of the increased cell titre. Additionally, Glx was not present in the fed-batch feed, resulting in a dilution effect from the medium addition.

As explained in Chapters 2 and 3, the depletion of Glx/Asn occurred at a similar point to the switch in lactate metabolism and cell growth from exponential to stationary phase. However, a switch in the lactate metabolism did not occur for the 140 mmHg fed-batch condition. This is likely to indicate that the feed conditions require optimisation, such as amino acid composition and feeding time; as other factors have an interacting role on the production of lactate, such as the osmolality increase. In the previous study (Chapter 3), elevated osmolality was shown to increase lactate levels.

(a)

(b)
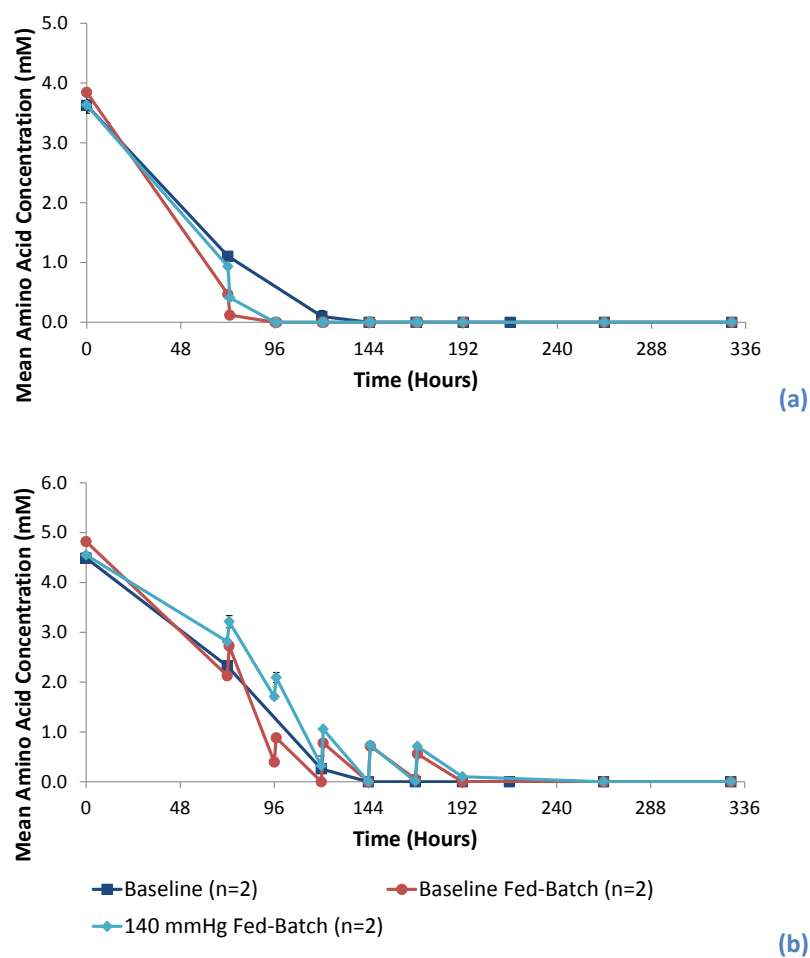
Figure 4.10 - L-alanine-L-glutamine (a) and Asparagine (b) Concentration Time Profiles for pCO$_2$ Control Conditions. (Note: Shown with S.E. of the mean)

For the fed-batch 140 mmHg conditions glycine was not consumed (Figure 4.11 (a)), unlike the 140 mmHg batch conditions in which glycine was consumed from 144 hours (Chapters 2 and 3). This may be due to the increased concentration of amino acids from the feed, meaning that glycine consumption was not required for the TCA cycle. These results support the conclusions reached in

Chapters 2 and 3 that process specifics and parameters can have an interacting effect on the results of the process parameters being investigated.
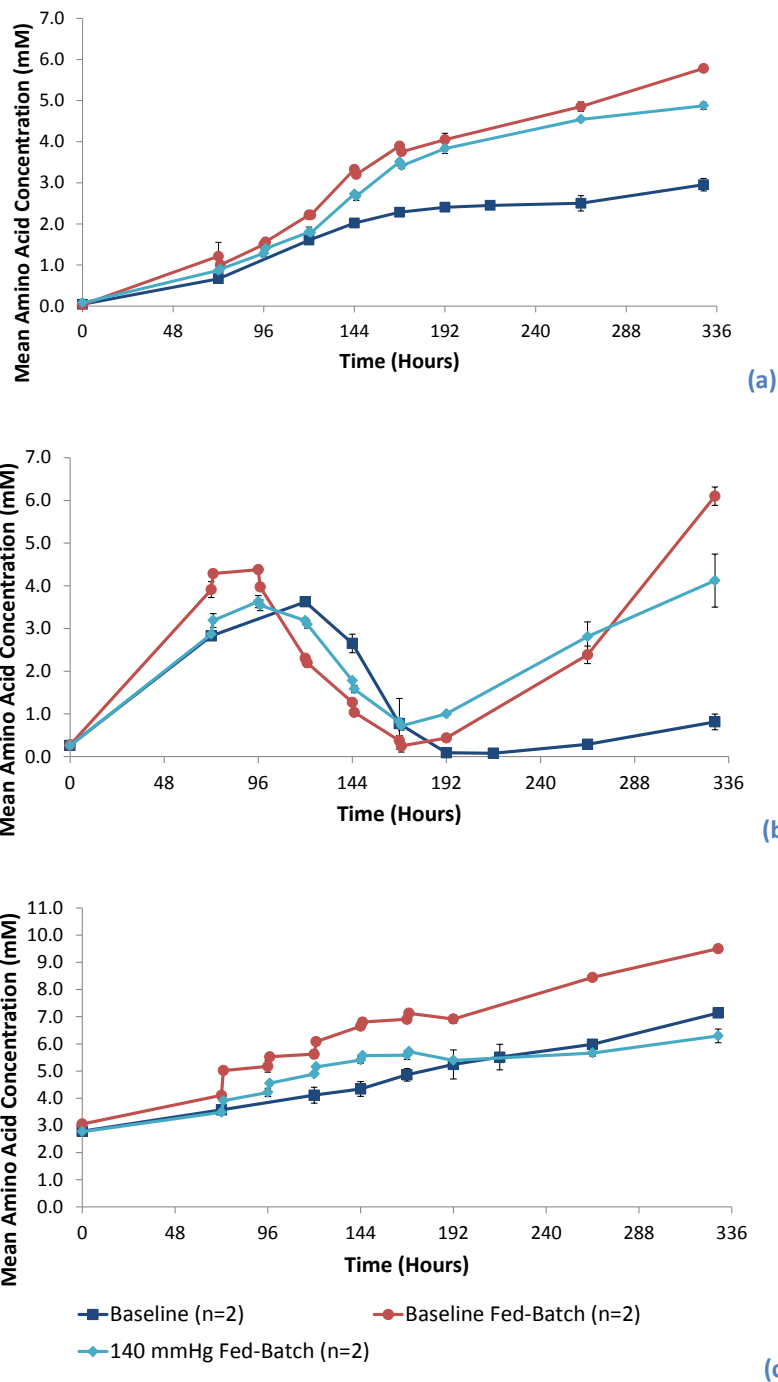
Figure 4.11 - Glycine (a), Alanine (b) and Glutamate (c) Concentration Time Profiles for pCO$_2$ Control Conditions (Note: Shown with S.E. of the mean)

In Chapters 2 and 3, a similar metabolism between the lactate and alanine was observed, with production during the exponential phase and consumption during the stationary phase. However, for the 140 mmHg fed-batch condition a decoupling of the alanine and lactate metabolism is

observed (Figure 4.6 and Figure 4.11). In all conditions the re-metabolism of alanine was observed, whereas for the 140 fed-batch condition lactate was not re-metabolised. Additionally, an increase in alanine concentration during the stationary phase was observed for the fed-batch conditions.

The increase in alanine concentration post 192 hours for the fed-batch processes is linked to the cell viability decline. It is likely that the increase is due to an increased pyruvate production from high concentrations of glucose and low TCA activity of dying cells, as well as the increased concentration of other available amino acids. Lactate and alanine are produced from pyruvate.

As observed in Chapters 2 and 3, glutamate is produced throughout the culture, shown in Figure 4.11 (c). The 140 mmHg fed-batch and baseline batch conditions had a similar glutamate condition, with an increased concentration for the baseline fed-batch condition, resulting in a 2mM increase in concentration. The results again indicate that the cell line does not consume glutamate during the culture.

4.3   Discussion

The study results show that under fed-batch conditions the control of pCO_2 affects the cell culture in terms of product quality and cell metabolism. However, due to an inefficient/non-optimised feed process, the cell viability declined rapidly. The inefficient feed process was highlighted by the build-up in aspartate concentration to above 20 mM. This indicates that the feed contained amino acids not required by the cell and the build-up to 20mM may have had an inhibitory/toxic effect. Additionally, glucose was added in a bolus shot, to 10 $g.L^{-1}$ during the decline phase (to prevent depletion over the weekend). The additional stress of the glucose and osmolality increase (due to glucose) may have also played a role in the rapid viability decline.

For future runs an optimised feed protocol or a custom amino acid feed would potentially prevent a build-up of amino acids and also the depletion of other key amino acids. The results indicate a number of issues including the evidence of interacting effects from the process, such as amino acid feed, on the response to pCO_2/osmolality. This supports the conclusions reached from the results attained in Chapters 2 and 3 that a number of parameters can have an impact on the response to process parameters investigated, i.e. the multivariate nature of cell cultures. The study results support that pCO_2 and osmolality levels should be considered in QbD studies as it can have a varied effect on the response of a cell culture process.

4.3.1 Productivity

Fed-batch operation gave rise to a similar mAb titre irrespective of the osmolality or pCO_2 level, shown in Figure 4.1. This is likely due to the amino acid concentration playing a more significant role or the fact that an un-optimal process masked the effect of pCO_2/osmolality. In order to determine if the lack of effect on product titre from pCO_2/osmolality is due to the non-optimised process, an optimised feed process is required. It is also recommended that an examination of the interacting effect between the feeding strategy and pCO_2/osmolality control level should carried out.

Optimisation of the process is required, as the combination of the commercial amino acid feed and bolus glucose feed led to non-optimal performance, for this study. For example, the commercial feed (in this study) contained high levels of Asp and Glu, of which this cell line consumed Asp at a slow rate and (net) produced Glu in the process. Therefore, there was a build-up of Asp and Glu in the culture. Additionally, the levels of glutamine (in the form of Glx) and asparagine (Asn) were not sufficient, as these were depleted at the same time as the batch culture. A more appropriate feed strategy would be to use one regime for the cell growth and one for the mAb production, as they have been shown to be separate cell cycles (Altamirano *et al*, 2001).

4.3.2 Product Quality

As observed in the previous studies (Chapters 2 and 3), pCO_2 control effects the product quality. Operation at 140 mmHg fed-batch led to improved target mAb over the baseline and baseline fed-batch condition. As there was no significant difference in the amino acid concentrations to account for the difference in product quality and hence the metabolism between the baseline and 140 mmHg fed-batch, it is likely that another factor contributed to the improved product quality. The likely cause is considered to be the effect of higher concentration of pCO_2 on the cell or the effect of pCO_2 on the pHi, as discussed in Chapters 2 and 3. However, the measurement of the pH_i is required to determine if the effect of pCO_2 on pH_i is the cause of the observed effect on product quality with pCO_2 level (as discussed in Chapters 2 and 3).

As there was little difference in the amino acid concentrations and hence the metabolism between the baseline and 140 mmHg fed-batch, it is likely that another factor contributed to the improved product quality. As discussed previously, the likely cause is considered to be the effect of pCO_2 on the internal pH or cell metabolism.

4.3.3 Cell Growth

The increase in viable cell concentration under the fed-batch condition is likely due to the increased amino acid concentration, resulting in increased nutrients for cell growth (Figure 4.6 and Figure 4.5). Additionally, from 0 to 96 hours the fed-batch baseline condition had a lower osmolality than the baseline batch, due to pre-loading of glucose for the batch condition (which increased the osmolality). Therefore, the higher growth may also be partially due to the reduced osmolality level.

For the 140 mmHg fed-batch condition a significant increase in cell titre was not observed. This is likely due to the non-optimised feed conditions, highlighted by the increased decline in cells for the 140 mmHg fed-batch condition. Despite the significant difference in viable cells between the fed-batch baseline and 140 mmHg conditions a very similar mAb titre was observed.

4.3.4 Cell Metabolism

The fed-batch conditions resulted in a number of changes to the cell metabolism compared to the batch condition. In general, the amino acid levels increased, which is expected as increased levels of amino acids have been added to the culture. However, there were no significant changes in the profiles of the cell metabolism (e.g. ammonium, Glx/Asn, etc.).

The most significant difference was seen in the lactate profile for the 140 mmHg fed-batch condition, where lactate was not re-metabolised. It is hypothesised that the increase in lactate is due to the significant increase in the osmolality (Figure 4.8). The osmolality increase was due to the combination of the amino acid feed and the addition of 1M NaOH to maintain the pH at the increased pCO_2 level. It is also possible that the increase in osmolality level is due to the elevated lactate levels (opposed to the cause), suggesting that the elevated lactate levels are due to another factor, such as the build-up of a cell culture component to inhibitory levels. From investigation of the amino acids profiles the only amino acid to increase to significantly high levels was the aspartate to levels above 20 mM in both fed-batch cultures (data not shown). To the author's knowledge, there is no literature on the effect of excessive aspartate feeding to CHO cell culture. For all fed-batch conditions the aspartate concentration exceeded 20 mM, therefore, there may be an interacting effect between the aspartate and pCO_2 control level. The reduced cell titre for the 140 mmHg fed-batch condition may have resulted in the toxic conditions, as the aspartate to cell titre ratio would be greater compared to the baseline fed-batch condition. Further investigation is required to determine the cause along with the optimisation of the feed process, however, as this would not affect the overall conclusion of the study, other runs were prioritised over this.

4.4 Conclusions and Future Work

The study results have demonstrated that pCO_2 control affects a fed-batch cell culture process, with a similar effect on product quality as was observed in the batch cultures in this study. Additionally, the investigation has demonstrated that other process parameters, such as amino acid feeds, have an interacting effect on the response to pCO_2 control level. Unlike the previous studies (Chapters 2 and 3), pCO_2 control level and osmolality level were shown to have no significant impact on the product titre. This indicated that for this cell culture process (cell line and media), the effect of amino acid concentration (i.e. feed) dominates the mAb production response. The process requires optimisation and should be repeated after the feed process has been optimised. However, even with optimisation of the fed-batch the main findings would not considered to differ, i.e. improved product quality observed at elevated pCO_2 in batch studies is also likely to be observed in under fed-batch conditions. Therefore, a fed-batch optimisation study was not carried out and the osmolality study and PCA work prioritised over this.

Control of pCO_2 affected the charge variant profile, as was seen in the batch conditions (Chapters 2 and 3). Fed-batch operation at an elevated pCO_2 control (140 mmHg) led to a significant increase in the target mAb composition, compared to both the batch and fed-batch baseline conditions. The increase in target mAb at the elevated pCO_2 under fed-batch conditions was more significant than observed under batch conditions. However, glycan and further product quality analysis were not carried out (due to cost) and should be undertaken to examine the effects of pCO_2 control and osmolality level on a number of product quality attributes.

The results demonstrated the sensitivity of the cell culture process to fed-batch operation, as has been previously noted with the current feed (data not shown). This shows that the effect of certain process parameters can be masked due to non-optimal conditions and highlights that during fed-batch process design, the consideration of all interacting parameters is required.

For future work, optimisation of the fed-batch process is required, which is a complex and extensive process. This should include the design of a base and feed media and feeding regime to prevent the depletion and build-up of amino acids required by the cell. Amino acid analysis has demonstrated that the feed media used contained too high levels of aspartate leading to a build-up, which is potentially inhibitory. Also, the interacting effect of pCO_2, osmolality and feeding strategy needs to be investigated in the form of a DoE. However, with the immense number of other interacting factors, which are difficult to or cannot be controlled, this may not be plausible for a dynamic cell culture system.

5 Multiway Principal Component Analysis of Mammalian Cell Culture Data

5.1 Introduction

The principal goal of process analytical technology (PAT) and Quality by Design (QbD) is to increase the understanding and quality of biotechnological and pharmaceutical manufacturing processes. Due to the large amount of measured data available from bioprocesses, particularly with the growing application of PAT, increasingly complex and correlated data sets are generated, which are difficult to interpret. Additionally, mammalian cell processes are inherently complex systems due to the highly correlated and interdependent cell metabolism, i.e. they are living cells which respond to stimuli. A set of methods to interpret the complex data sets, recommended by the regulators as a key enabler for PAT and QbD, is multivariate data analysis (MVDA).

Multivariate data analysis is essential to extract information from complex data sets. Large data sets can be challenging and time consuming to analyse and relationships may be missed if only univariate analysis is applied. The aim of MVDA in industry is to maximise the use of process data in order to increase process understanding and improve process efficiency and quality. There are a number of MVDA techniques available for data analysis, with one of the most popular and powerful being Principal Component Analysis (PCA) (Glassey, 2013; Gunther *et al*, 2007; *Huang et al*, 2009). PCA can be applied to historical data allowing it to be used alongside or independently of DoE studies.

There have been limited examples published on the application of MVDA for biopharmaceutical data, particularly for PCA and bioreactor cell culture data. This is mainly due to the complex nature of the bioprocesses, early adoption of MVDA techniques and the fact that the analysis is performed on confidential production data. MVDA has been widely applied to data from a range of industries including food, pharmaceutical, chemical and environmental, with very limited publications on its use in the biopharmaceutical industry. Principal component analysis is typically the first step in MVDA and is used to study the structure of data to identify trends or clusters within the data, with the aim of providing greater understanding of the data set.

5.2 MVDA in the Biopharmaceutical Industry

MVDA has been applied upstream, downstream and a combination of both in the biopharmaceutical industry. For this review, mainly the application of PCA to upstream cell culture operations, i.e. bioreactor cell culture data, will be discussed, as this is considered as the most relevant.

The importance of MVDA to support QbD for drug development was demonstrated by Huang *et al* (2009), in which PCA was applied to study the relationship between variables available from a DoE campaign. Huang *et al* demonstrated that both PCA and Partial Least Square regression (PLS) could be used to extract and understand complex multivariate relationships between batches and also between variables. The study highlighted that PCA and DoE are complimentary and more useful when used in combination.

Gunther *et al* (2007) applied PCA to a fed-batch recombinant production process, examining the model fit through the analysis of Hotelling T^2 and squared prediction error statistics in an attempt to detect and diagnose abnormal process conditions using offline and online (process) analysis. Gunther *et al* demonstrated that abnormal process conditions could be identified through the use of PCA. Although the non-optimal batches were highlighted as different, it confirmed what had been previously observed by plant operators. One example was a DO control issue, but, in practice these set-point deviations can be determined from a time series analysis. The study did demonstrate the potential for the application of PCA online for early fault detection and provided statistical support for observations made by plant engineers from the examination of time series plots. It should be noted that only operational data was collected, such as pH, temperature, etc. Therefore, only the relationships between operational parameters could be determined, as no biological process performance and product quality was recorded.

Nucci *et al* (2010) applied PCA online for fault detection in a fermentation process via online instrumentation. Detection of abnormal data during the experiments was possible using an online PCA algorithm. However, only online data was included in these models, therefore, process knowledge relating to biological variables was not captured. Additionally, for a well-controlled process, these set-point deviations would be evident from inspection of univariate process charts and from process alarms; therefore, the application of PCA potentially overcomplicated the process. The advantage of applying online PCA would be if two control parameters, which are within the individual set-points limits, result in non-optimal performance due to the issue being caused by the interaction between the set-points.

The papers of Gunther *et al* (2007) and Nucci *et al* (2010) made use of PCA for fault detection of processing monitoring equipment. However, very limited process knowledge could be extracted from these PCA representations, due to a lack of key variables, such as biological variables (cell metabolism, cell growth, etc.), being considered. They demonstrated the use of PCA as a potential monitoring tool, but did not illustrate the more powerful side of the methodology in terms of

indicating process interactions to determine what affects process performance and hence increase process knowledge.

A number of studies have combined online measurements with offline data, such as cell density and product concentration to create more informative PCA models than that discussed previously.

Mercier *et al* (2013) applied PCA to examine process data in an attempt to identifying causes for batch deviations and examine if there were differences between production scales at 2 and 10 L. PCA highlighted new relevant process information in this study, such as differences in the gas volume flow per unit of liquid volume per minute (VVM) between 2L and 10L scales, which led to differences in performance. Additionally, PCA was used to identify deviating batches and led to targeted analysis of the cause of the deviations.

Ferreira *et al* (2007) used PCA to detect differences between batches and for the identification of abnormal variability in quality variables for a large data set generated from an industrial pilot-scale fermentation process. Cunha *et al* (2002) applied PCA to an industrial fermentation process to assess the quality of seed batches and to extract distinguishing features between high and low productivity batches.

Green and Glassey (2014) examined the effect of operating conditions on the cell metabolism and glycosylation pattern of a monoclonal antibody produced in a hybridoma cell culture. PCA analysis indicated significant dependence on operating conditions. Partial least square regression (PLS) was also used to model the product quality and quantity; however, the model fits were poor due to lack of included process parameters. The product titre modelled was below 0.1 $g.L^{-1}$, which is significantly lower than industrially achievable titres of greater than 1 $g.L^{-1}$. Therefore, the fit of the model would likely be significantly poorer when applied to a higher titre processes. The study highlighted that when insufficient data was included in the model, such as only online data, a poor model is produced. This highlights the need to capture the relevant information in the model, which includes biological parameters, such as cell metabolism related variables.

Schuab *et al* (2012) used PCA as part of process development for a recombinant product CHO process. Nine high performing culture runs from a total set of 45 runs were examined using PCA. The results showed clustering of the data into three groups; however, the cause(s) of the clustering were not discussed.

PCA has also been applied to a number of cases aside from dimension reduction for process knowledge, such as multivariate calibration models (e.g. NIR, MIR, Raman) for on-line monitoring of bioprocess and for statistical process control (Gnoth *et al*, 2008; Teixeira *et al*, 2009).

Studies have shown the application of PCA to increase process knowledge for the biopharmaceutical industry. However, little was discussed on the interoperation of the PCA data and the resulting knowledge learnt from the analysis; rather the actual application of the PCA to the data has been presented in literature. There is limited literature on the use of PCA for upstream bioprocess applications, particularly for the analysis of cell culture data. In this Chapter, data generated from previous studies (Chapters 2 and 3) will be analysed using PCA. The aim of this study is to increase process knowledge and to demonstrate the application of PCA to bioreactor cell culture data. The use of PCA for data analysis is of high interest to Pall Life Sciences, as a large amount of historical data is generated, therefore, a secondary aim of the PCA is to introduce and demonstrate the use of PCA to Pall.

5.3   Principal Component Analysis

PCA reduces the dimensionality of a data set through the projection of correlated process variables onto a new plane which is defined in terms of a smaller set of latent variables. These latent variables (principal components) are a linear combination of the process variables and their corresponding weights (loadings). The first few principal components (PCs) capture most of the variability of the data set and hence the data set can be explained using fewer PCs, without a significant loss of information. The first PC explains the greatest level of variability in the data with each successive component describing the remaining greatest variability and being orthogonal to the previous component. The main aim of PCA is dimensionality reduction, i.e. to reduce the number of variables for analysis, to enable the delivery of enhanced process understanding.

5.3.1 PCA Decomposition

The objective of PCA is to decompose a data matrix $\boldsymbol{X}$ (m rows and n columns) into a structure part and noise part:

$$\boldsymbol{X} = \mathrm{TP}^{\mathrm{T}} + \mathrm{E} \qquad\qquad \textbf{(7)}$$

$$\boldsymbol{X} = Structure + Noise$$

The data matrix **X** is split into a sum of a matrix product, **TP**$^{\mathbf{T}}$, and a residual matrix **E**. **T** is the score matrix and **P**$^{\mathbf{T}}$ is the accompanying loading matrix (which is transposed). The aim of PCA is to determine **T** and **P** and use their outer produce instead of the original data matrix **X**, with the

remaining part of X which cannot be accounted for described as the error component, **E**. Equation 6 can be written as the individual PC contributions, as individual vector products (Azmin, 2013):

$$X = t_1 p_1^T + t_2 p_2^T \dots + t_r p_r^T \dots + t_R p_R^T + E \qquad (8)$$

The number of components retained is R, which must be less than or equal to the smaller dimension of X (i.e. r ≤ min[m, n]). The score vector (t_i) is a linear combination of the original X as variables defined by the loadings vector (p_i).

$$t_{id} = x_{ij} p_{jd} \qquad (9)$$

Where x_{ij} is the element of the jth variable measured for the ith sample and p_{jd} is the vector of loadings for variables j in dimension d (Azmin, 2013). The loading vector (p_i) contains the information of the relationship between variables and is defined as the eigenvector of the covariance matrix of X:

$$cov(X) = \frac{X^T X}{n-1} \qquad (10)$$

The p_i vectors are eigenvectors of the covariance matrix and for each p_i:

$$cov(X) p_i = \lambda_i p_i \qquad (11)$$

Where λ_i is the eigenvalue associated with the eigenvector p_i. λ_i is a measure of the variance explained by each principal component (Azmin, 2013).

PCA Associated Statistics

A lack of model fit statistic, Q, can be calculated for PCA models, which describes the variation not captured by the model:

$$Q_i = e_i e_i^T \qquad (12)$$

where e_i is the ith row of E. The sum of normalised squared scores is known as Hotelling's T^2 and is a measure of the variation in each sample within the model and is defined as:

$$T_i^2 = t_i \lambda^{-1} t_i^T \qquad (13)$$

where t_i is the ith row of T, the matrix of r scores vectors from the PCA model and λ^{-1} is the diagonal matrix containing the inverse of the eigenvalues associated with the r eigenvectors (PCs) retained in the model.

The Q and Hotelling's T^2 statistics are used to assess the fit of the model and can be used to examine for non-conforming data points.

Contribution Plots

Contribution plots are used to provide an insight into which variables are responsible for the positioning of individual scores on a specific PC. The loading is the sum of the product of the original data and loadings across all time points. Therefore, for each PC the contribution is (Molloy and Martin, 2013):

$$C_{ij} = \sum_{k=1}^{k} x_{kij} p_j \qquad \textbf{(14)}$$

where C_{ij} is the contribution for run i and variable j on the chosen PC. p_j is the loading for the given variable and $\sum_{k=1}^{k} x_{kij}$ is the sum of the original (autoscaled) data matrix across all time points for a given batch and variable.

Scores and Loadings Plots

'Scores' describe the relationships between samples and the 'loadings' describe the relationships between variables in the model. Bivariate scores plots illustrate the samples projected onto the new principal component planes and can reveal patterns and grouping of samples. The scores plots can be used in conjunction with the variables loadings by observing changes in the scores and relating them back to the variable loadings.

Univariate loadings plots demonstrate how the variables contribute to defining the orientation of the principal component. Bivariate loadings plots demonstrate the relationships between variables and can be used to interpret patterns observed in the scores plot. Additionally, bivariate loadings plots can be used to identify correlations between variables.

Overlaying the scores and loadings plot results in a biplot. The biplot gives a visual representation of which variables are responsible for the positioning of the sample scores on the PC plane, i.e. samples located close to a variable in the PC plane demonstrate a significant impact from that variable.

5.4 Analysis Aim

The aim of this Chapter is to demonstrate the approach of Principal Component Analysis to cell culture data, as both an exploratory data analysis tool and how it can enhance process knowledge. Additionally, the information gained can be used in the design and development of future processes. The outcome of the PCA is to increase process understanding through the identification of key parameters and the identification of variable relationships and, in particular, the change in these parameters across the pCO_2 control levels. Secondly, the aim is to demonstrate the application of PCA to cell culture data (particularly for Pall), as there are limited publications on the approach of MVDA for upstream bioprocessing applications.

PCA was applied in this study, with the aim of extracting further understanding from the data sets discussed in Chapters 2 and 3. A data set comprising 40 STR runs across seven batches was collected to examine the effect of pCO_2 control and osmolality level on a mammalian cell culture process. For this context a 'run' is an individual STR culture and a 'batch' is a group of STR runs carried out from the same inoculum at the same start time. The data set is summarised in Table 5.1.

Batch Number	pCO$_2$ Level (mmHg)	Osmolality Control	Runs in Batch (n)
1	Baseline	-	2
	60	-	1
	100	-	1
2	Baseline	-	2
	20	-	1
	60	-	1
	100	-	1
3	Baseline	-	1
	20	-	1
	60	-	1
	100	-	1
	140	-	2
4	Baseline	-	1
	20	-	1
	100	-	1
	140	-	2
5	Baseline	-	1
	20	-	2
	20	High	2
	140	-	1
6	Baseline	-	1
	Baseline	High	1
	Baseline	Low	1
	140	Low	2
	140	-	2
7	Baseline	High	2
	Baseline	Low	1
	140	Low	2
	140	-	2

Table 5.1 - STR Test Conditions showing the conditions carried out in each of the 7 batches conducted

In the studies presented in Chapters 2 and 3, there are over 50 potential process variables to examine within a single STR condition, with each sampled for a minimum of 14 time points (up to 21600) carried out across 9 different conditions. Therefore, examining the data set becomes difficult due to the size and multivariate relationships between variables. Subsequently, data trends can easily be missed using univariate data analysis. Additionally, cell culture data is a highly multivariate process, therefore examination of the data set becomes difficult. Application of PCA to the data set results in dimensionality reduction, i.e. the transformation of the data set to examine it using new fewer variables. The entire data set can be examined using fewer plots with correlations and outliers identified.

The objective of the experimental work was to examine if pCO_2 was a critical process parameter by investigating its effect on the critical quality and performance attributes, specifically productivity and product quality, of a mAb producing CHO cell line. The conditions investigated and the variables collected are listed in Table 5.1 and Table 5.2, respectively.

Within each batch, a baseline condition was carried out as a blocking point to make comparisons between batches (as explained previously in Chapter 2). The baseline condition is a standard cell culture operating condition, chosen to represent a standard batch cell culture process.

Variable	Method	Frequency
pH	At-line, Flex Autosampler	24 Hours
pO_2	At-line, Flex Autosampler	24 Hours
pCO_2	At-line, Flex Autosampler	24 Hours
Glutamine	At-line, Flex Autosampler	24 Hours
Glutamate	At-line, Flex Autosampler	24 Hours
Glucose	At-line, Flex Autosampler	24 Hours
Lactate	At-line, Flex Autosampler	24 Hours
Ammonia	At-line, Flex Autosampler	24 Hours
Sodium	At-line, Flex Autosampler	24 Hours
Potassium	At-line, Flex Autosampler	24 Hours
Calcium	At-line, Flex Autosampler	24 Hours
Osmolality	At-line, Flex Autosampler	24 Hours
Total Cell Density	At-line, Flex Autosampler	24 Hours
Viable Cell Density	At-line, Flex Autosampler	24 Hours
Viability	At-line, Flex Autosampler	24 Hours
Cell Diameter	At-line, Flex Autosampler	24 Hours
mAb Titre	Offline, HPLC Protein A	3 Times Weekly
Amino acids (x22)	Offline, UPLC Derivatisation	3 Times Weekly
Charge Variants	Offline, UPLC Cation Exchange	3 Times Weekly
Online pH	Online, Sensor	Once per Minute
Online pCO_2	Online, Sensor	Once per Minute

Table 5.2 - Measured Variables and Frequency Used in the Study

The work discussed in Chapters 2 and 3 showed that pCO_2 control affects the productivity, product quality and cell metabolism of a cell culture process. In summary, increasing pCO_2 control from 20 to 140mmHg, leads to increased productivity, compared to the baseline. Increasing pCO_2 control levels from 20 to 100mmHg resulted in worsening product quality (defined by the charged variant profile); however, at 140mmHg the product quality was equivalent to that of the baseline. Therefore, operation at elevated pCO_2 control (140mmHg) results in increased productivity with the maintenance of product quality, relative to the baseline condition.

The two aims of PCA in this study are to interrogate the data further to illustrate the cause of the difference between control levels and also to present an overall picture of the data in a multivariate format.

5.4.1 Software

Data pre-processing and PCA were carried out using MATLAB (Version 7.10.0.499, The Mathworks Inc.) and the PLS toolbox (Version 6.2.1, Eigenvector Research Inc., Wenatchee, USA).

5.4.2 Data Pre-Processing

Due to irregular sampling intervals between online sensors, at-line samples and offline analytics, samples were interpolated to new equal time points via linear interpolation to give a sampling rate of 12 hours. Linear interpolation was justified due to the strong trends displayed in the data. The limitation of interpolating the data is that subtleties in the online data may be lost. Additionally, due to the low sample rate for the offline assays, interpolation may introduce errors in the amino acids and mAb titre values, as these have comparatively few sample points. Therefore, caution is required when drawing conclusions from these variables and possible correlations may or may not be captured.

5.4.3 Data Unfolding

Batch data can be considered as a three dimensional data set, process variables (j) by time (k) by STR run (i). Principal Component Analysis is a method to decompose two dimensional data sets, therefore, an extension to PCA is required to analyse three dimensional data, known as multiway PCA (Nomikos and MacGregor, 1994; Wold *et al,* 1998).

Multiway PCA (MPCA) unfolds a three dimensional data set into two dimensions, so that a traditional PCA decomposition can be performed. There are a number of methods for unfolding the data set, which allow for different interpretation of the results. The two methods used for this study were the Nomikos and MacGregor (1994), i x jk and Wold *et al* (1998) ik x j methods.

The first method described by Nomikos and MacGregor (1994), results in an unfolded data matrix of i x jk. This method allows for a comparison of batches around the mean trajectory, i.e. examining deviations about the mean. The advantage of this method is that the mean trajectory is removed (which would otherwise dominate the model) to allow for the examination of the underlying behaviour or differences between batches. In this method STR runs are represented as scores and loadings the variables over time. The structure of the unfolded data matrix i x jk is shown in Figure 5.1. For this method each score is an entire STR run.

	Variable 1			Variable 2			Variable j		
	Time1	Time2	Timek	Time1	Time2	Timek	Time1	Time2	Timek
Run1									
Run2									
Run i									

Figure 5.1 - Representation of Data Unfolded Using the Nomikos and MacGregor Method

The second unfolding method, as described by Wold et al (1998), results in a ik x j matrix. This method allows for examination of the data across the culture time, with each sample score being a time point of a cell culture run and a loading an individual variable. The structure of the unfolded data matric ik x j is shown in Figure 5.2.

		Variable 1	Variable 2	Variable j
Run 1	Time 1			
	Time 2…			
	…Time k			
Run 2	Time 1			
	Time 2…			
	…Time k			
Run i	Time 1			
	Time 2…			
	…Time k			

Figure 5.2 - Representation of Data Unfolded Using the Wold Method

Autoscaling

The data was scaled and mean centred, globally, by subtracting the mean and dividing by the standard deviation for each column. The purpose of the scaling was to allow for comparison between variables with differing ranges, for example lactate ranged from 0-3 g.L^{-1} whereas dissolved oxygen (DO) ranged from 40-100 %. Mean centring allows for the analysis of the variation between variables, by aligning the data.

Model Variables

Based on prior process knowledge and iterative exploration whilst creating PCA representations, variables best representative of process behaviour were included. Variables known to have little or no effect relating to pCO$_2$/osmolality control, such as amino acids which are consumed at a similar rate irrespective of control level, were removed. Other parameters which were tightly controlled and had little difference across pCO$_2$ levels, such as DO and temperature, were also removed. Ideally air, O$_2$ and CO$_2$ flow rates would have been included in the model. However, due to failure in the data logging system, gas flow rates for a number of batches was missing, therefore, they were removed for all batches. The variables included in the model are shown in Table 5.3.

Acronym	Included Variable
Ala	Alanine
Asn	Asparagine
Avg. Live Diameter	Average Live Cell Diameter
Gln	Glutamine
Glu	Glutamate
Glx	Ultraglutamine
Gly	Glycine
Lac	Lactate
mAb Titre	mAb Titre
Na+	Sodium
NH4+	Ammonium
Norm mAb	Normalised mAb Titre
On_pCO$_2$	Online pCO$_2$
On_pH	Online pH
Osmolality	Osmolality
Viability	Viability
Viable Density	Viable Cell Density

Table 5.3 - Included Variables in PCA Model

5.5 Results and Discussion

Initially, the data was analysed using the unfolding method described by Nomikos and MacGregor (1994), with a score denoting a specific STR run, to examine for outliers and differences between STR runs. The unfolding method described by Wold *et al* (1998) was then applied to the data to study the time progression of the STR runs and to identify differences in behaviour. In this case each sample score was a time point of a specific STR run. Finally, the data was split into the exponential and stationary phases and further examined using the Wold method. The rationale for considering the two phases separately was to enable the incorporation of the product quality data, which could only be measured during the stationary phase. Additionally, the exponential and stationary phases can be considered as two separate cell stages and therefore, it is hypothesised that more information could be extracted from the data by treating the two phases separately.

5.5.1 MPCA Model - Nomikos and MacGregor Method

The data was unfolded using the Nomikos and MacGregor method to identify if differences existed between batches. Autoscaling of the data results in the removal of the mean trajectory, allowing the model to describe the deviations about the mean. A summary of the model is given in Table 5.4.

Principal Component	Eigenvalue of Cov(x)	% Variance	% Cumulative Variance
1	1.55e2	36.36	36.36
2	6.31e1	14.77	51.13
3	5.63e1	13.18	64.31
4	3.42e1	8.01	72.33
5	1.86e1	4.35	76.68
6	1.64e1	3.84	80.52

Table 5.4 - Nomikos and MacGregor MPCA Model Summary

Using cross-validation (a method for calculating the number of PCs), 4 PCs were suggested; however, 6 PCs were retained. Examining the eigenvalues versus PC plot (Appendix 8.4.1), an elbow point at 5/6 PCs was identified. Selection of 6 PCs captures 80% of the cumulative variability, which aligns with the general rule of thumb for selecting the number of PCs that explains 80-90% of the variability (Azmin, 2013).

As the PCA model is being used with the goal of data mining as opposed to process monitoring, the inclusion of further PCs is not detrimental to the objective of the analysis. Selection of additional PCs may result in noise being included in the model. However, this would result in the scores and loadings plots of the highest PCs showing no trends or groupings, so the analysis is not affected. If the purpose had been to construct a model for online monitoring, then more caution would be

required to avoid capturing noise in the model. The next analysis step after selecting the number of PCs is to examine the model fit statistics, i.e. the Q residuals and Hotelling T^2.

Examining the Q residuals versus Hotelling T^2 plot, Figure 5.3, indicates that there are no STR runs exhibiting significantly different behaviour. This is due to the overall time related structure remaining the same for all conditions, which is the main source of variation. For example, all conditions displayed exponential cell growth with a stationary/death phase and lactate production phase followed by re-metabolism.
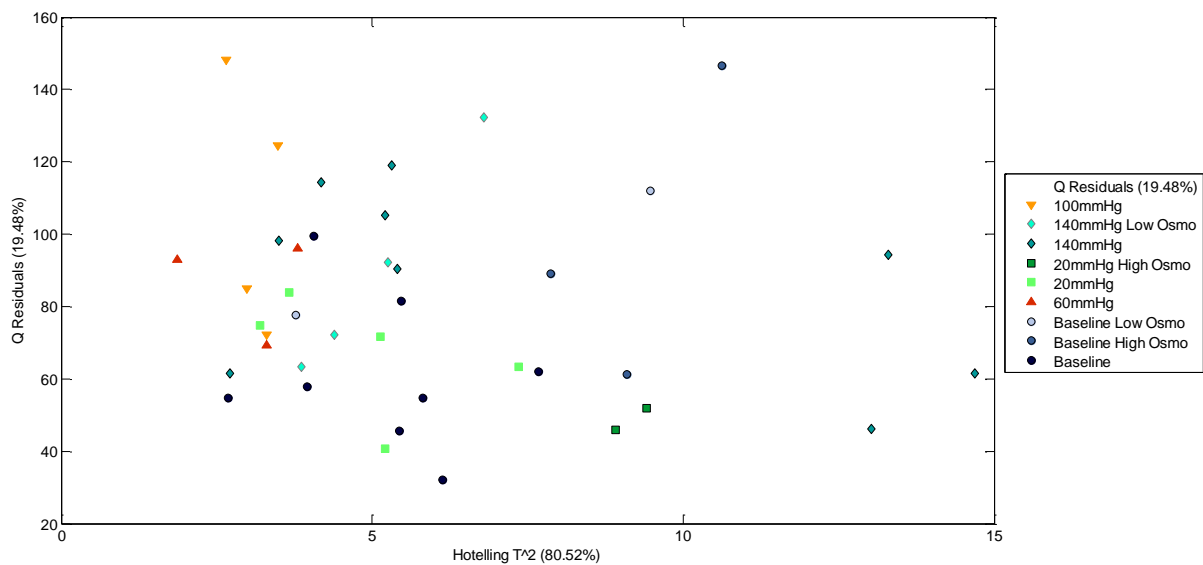
Figure 5.3 - Q Residual versus Hotelling T2 plot for Nomikos and MacGregor MPCA Model

PC2 versus PC1

The initial data analysis step involves the investigation of the structure of the data set, by examining the scores plot. Examining the scores plots for PC1 versus PC2, Figure 5.4, shows clustering of similar batches, based on pCO_2 control.
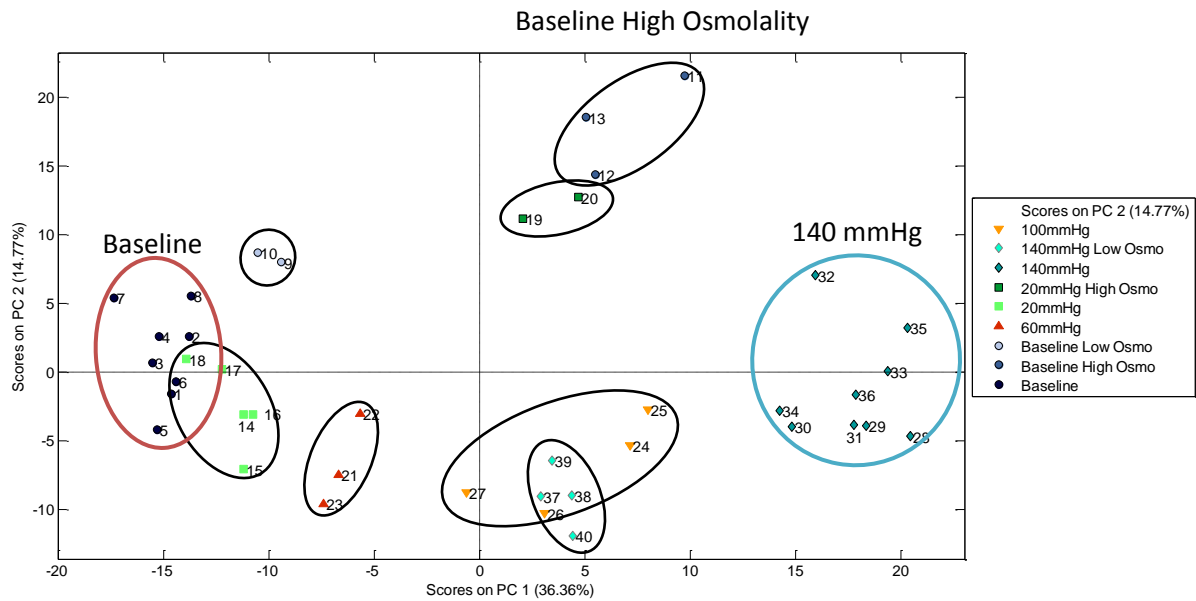
Figure 5.4 - PC2 versus PC1 Score Plot for Nomikos and MacGregor MPCA Model (IxJK)

The variation due to pCO_2 control level is mainly captured by PC1, shown in Figure 5.4 by the separation of batches on PC1, relating to pCO_2 control. Therefore, the plot indicates how the control of pCO_2 at different levels leads to clear differences in process parameters and is the main cause of the variability in the data.

The grouping of control conditions in the scores plots for PC2 versus PC1 can be understood using the univariate loadings plots for PC1 and PC2 and relating them to the time series plots. The positioning of the 140 mmHg conditions in Figure 5.4 compared to the baseline are considered to be due to a greater positive influence from the variables pCO_2, lactate, osmolality/sodium, alanine and mAb and strong negative influence from the glycine and viable cells variables. This aligns with the results discussed in Chapters 2 and 3 where the 140 mmHg conditions had higher levels of lactate, osmolality/sodium, alanine and mAb and lower levels of glycine and viable cells.

The univariate loadings plot for PC1 and PC2, Figure 5.5 and Figure 5.6, indicate how the variables impact on the position of the scores for the control conditions in Figure 5.4. For example, Figure 5.5 illustrates that lactate has a positive loading on PC1. Therefore, examining the scores plot indicates that the original data for lactate, for the control condition, has a more positive impact on the scores located positively on PC1. Examination of the scores and loadings plots (Figure 5.4 and Figure 5.5) alongside the times series plot indicates that scores with a positive value on PC1, such as the 140 mmHg conditions, have greater lactate levels than scores with a negative value on PC1, such as the baseline, as discussed in Chapters 2 and 3. Therefore, one of the causes of the separation of

conditions in the east of the plot is due to higher lactate levels, with lower level for the conditions located on the west of the plot. The same can be said for the other variables with a strong positive loading on PC1, Figure 5.5, such as sodium and osmolality.
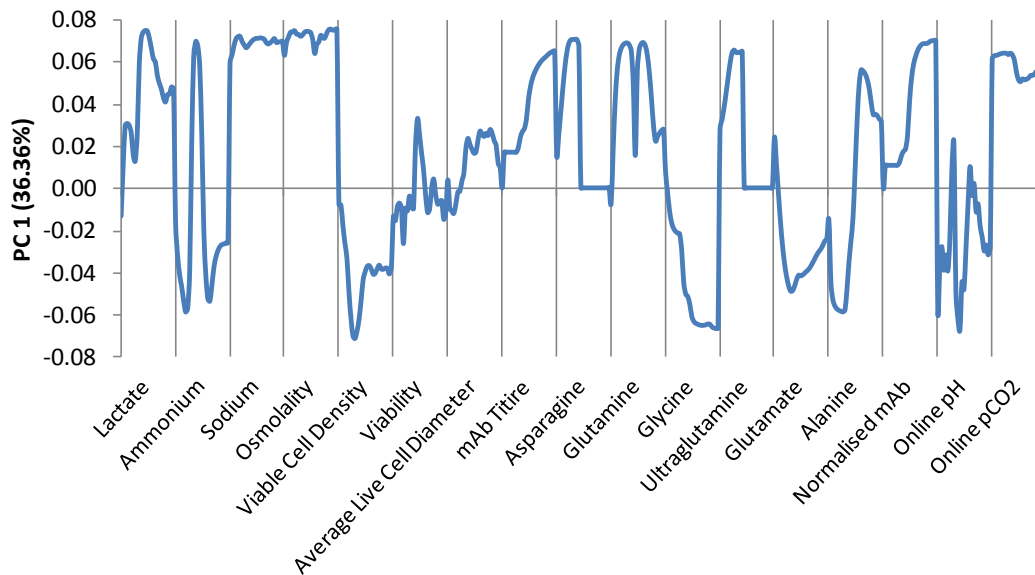
Figure 5.5 - Univariate Loadings Plot for PC1 for Nomikos and MacGregor MPCA Model
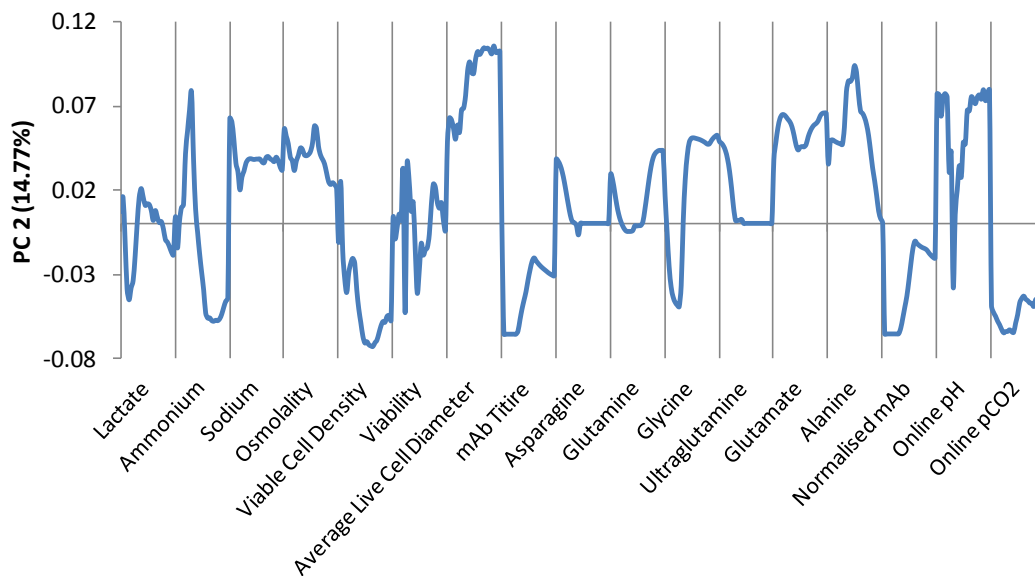
Figure 5.6 - Univariate Loadings Plot for PC2 for Nomikos and MacGregor MPCA Model

In addition to analysis of the univariate loadings plot, contribution plots can be used to summarise the total effect of the variables (loadings) on the position of the scores, providing an insight into which variables are responsible for the positioning of scores on a specific PC.

The contribution plot on PC1 is shown in Figure 5.7, for a baseline and 140 mmHg condition. Figure 5.7 indicates that a number of variables results in a separation of the scores on PC1 for the baseline

and 140 mmHg conditions. The main contributing variables are osmolality, sodium, pCO_2, glutamine, lactate and glycine, indicated by the larger contribution compared to the other variables. As the time series plots indicated in Chapters 2 and 3, these are the main differences between the test conditions. Therefore, the contribution plot can be used to identify the difference in variables relating to pCO_2 control level to give an indication of further analysis. The plot also demonstrates which variables exhibit similar behaviour between the conditions, such as ammonium, culture viability and average live diameter, shown by the small contributions, relative to the other parameters.
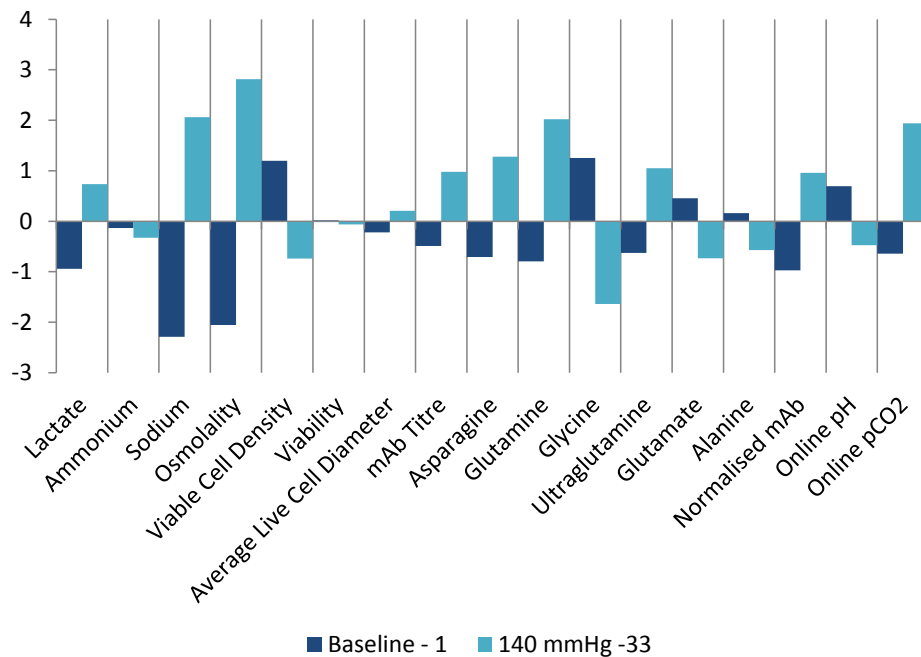
Figure 5.7 - Contribution Plot for Baseline (run 1) and 140 mmHg (run 33) Conditions for Nomikos and MacGregor MPCA Model

A separation of the high osmolality conditions from the other control conditions is shown on Figure 5.4, the scores plot of PC2 versus PC1. The separation of runs on PC2 is due mainly to average live cell diameter and alanine, as shown in the contribution plot on PC2 for an example baseline (run 1) and baseline high osmolality condition (run 11), Figure 5.8. The time series plots for average live cell diameter and alanine concentration, Figure 5.9, indicate increased levels for the high osmolality conditions. In summary, these results indicate that the elevated osmolality leads to an increase in cell size, which is a potential cause of the increased productivity noted with elevated osmolality. Additionally, elevated osmolality resulted in a decrease in the alanine consumption rate during the stationary phase, which may be due to the reduced cell density at high osmolality levels (as discussed in Chapters 2 and 3).
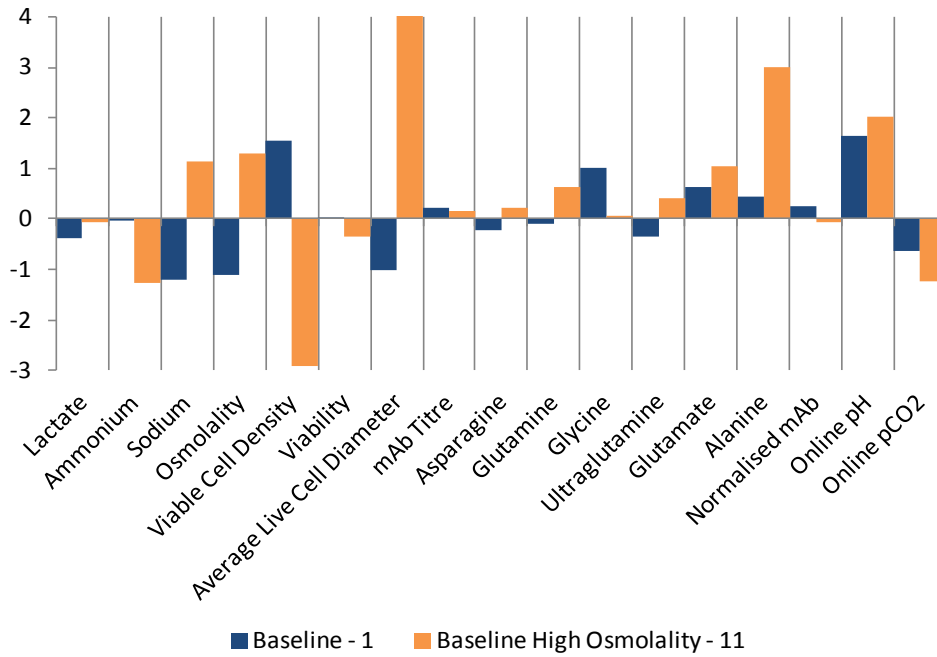
Figure 5.8 - Contribution Plot for a Baseline (Run 1) and Baseline High Osmolality (Run 11) Conditions on PC2 for Nomikos and MacGregor MPCA Model

(a)

(b)
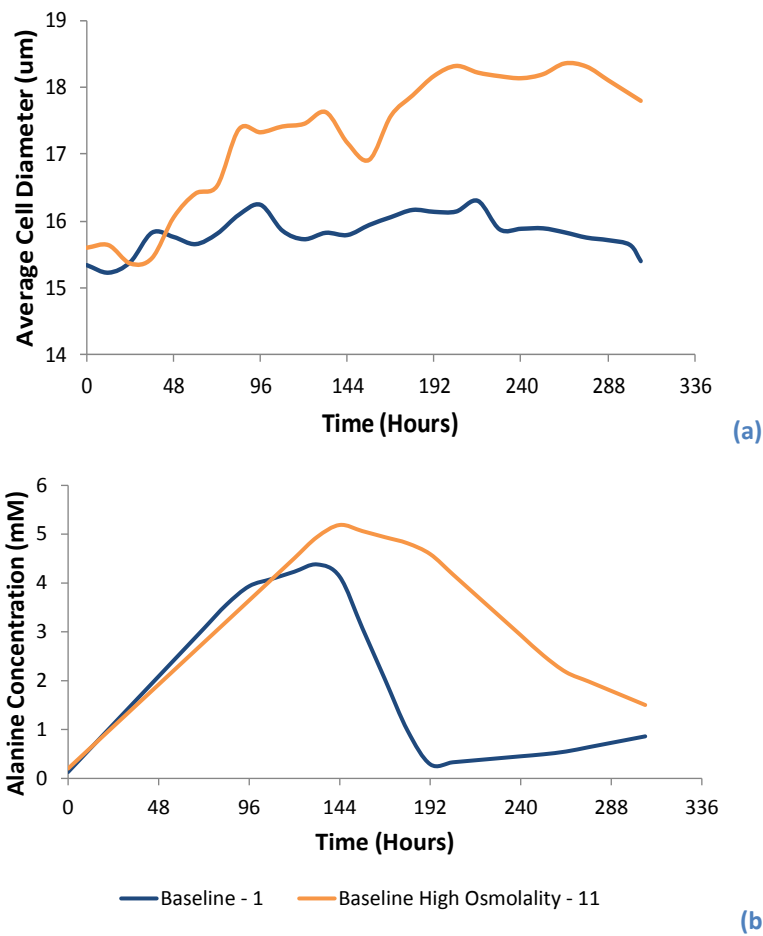
Figure 5.9 - Average Cell Diameter (a) and Alanine Concentration (b) for a Baseline (Run 1) and Baseline High Osmolality (Run 11) Condition

The close grouping of the baseline high osmolality and 20 mmHg high osmolality conditions in the scores plot for PC2 versus PC1, Figure 5.4, on PC1 indicates similar performance and hence highlights the importance of osmolality in cell culture, as adjusting the osmolality to the same value resulted in similar performance. These plots demonstrate how PCA can be used to summarise similarities and differences in the performance of STR runs and gives an indication of which parameters to examine, using time series plots.

PC4 versus PC3

The first and second PC explain the greatest amount of variation with subsequent PCs capturing lower levels of variation. Therefore, examination of higher PCs can yield more subtle differences between scores, such as PC4 versus PC3, Figure 5.10. A separation of two 140 mmHg runs (28 and 29) to the other 140 mmHg runs on PC4 is evident in Figure 5.10. Inspection of the contribution plots, Figure 5.11, between the 29 condition and one of the other 140 mmHg conditions, run 31, on PC4 shows the cause of the separation.
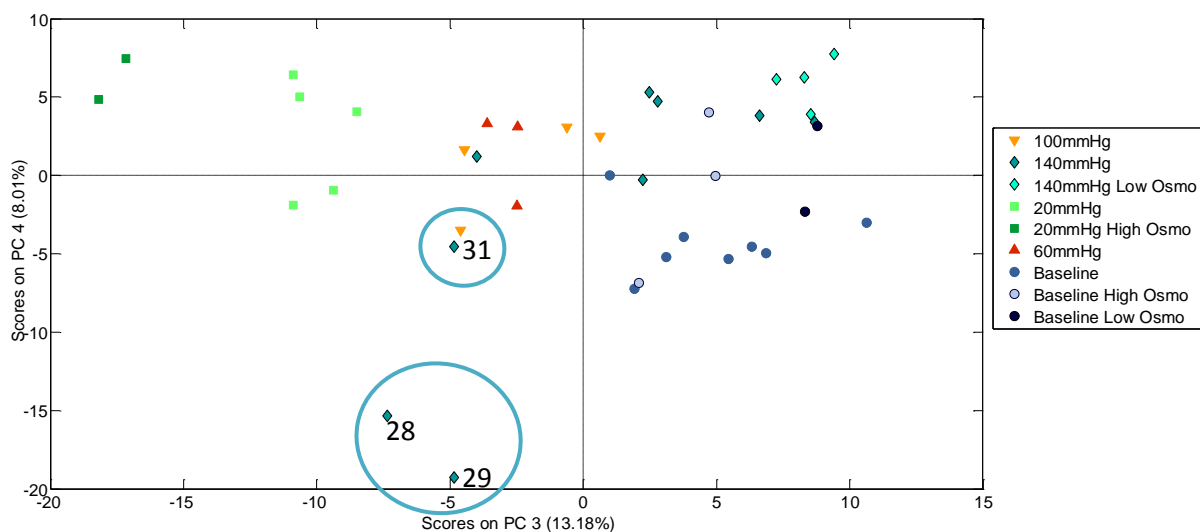
Figure 5.10 - Scores for PC4 versus PC3 for Nomikos and MacGregor MPCA Model

Figure 5.11 indicates that run 29 has a stronger contribution from alanine, average cell diameter, ammonium and lactate. Examining the time series plots indicates a smaller cell diameter for run 29 compared to run 31 (Figure 5.12) as well as increased alanine and lactate levels during the stationary phase (Figure 5.13), which coincides with lower ammonium levels. Increased lactate and alanine concentrations for run 29 suggests that there is a build-up of pyruvate in the culture, (a precursor of lactate and alanine) suggesting a slowdown in the TCA cycle. However, the TCA cycle is still active as product titre increases.

The cause of the increased lactate and alanine levels is unknown and indicates a change in the internal cell metabolism. A possible cause in the change in cell metabolism is the effect of clone line instability. A difference between runs 28 and 29 compared to the other 140 mmHg conditions was that the runs were carried out at a higher passage number. From the above results, it is suggested that passage number (i.e. clone stability) effects more than just the mAb titre. It could be concluded that passage number is a potential critical process parameter (for this process) and that further studies are required to confirm this conclusion, which is currently under investigation at Pall by the R&D Applications team.
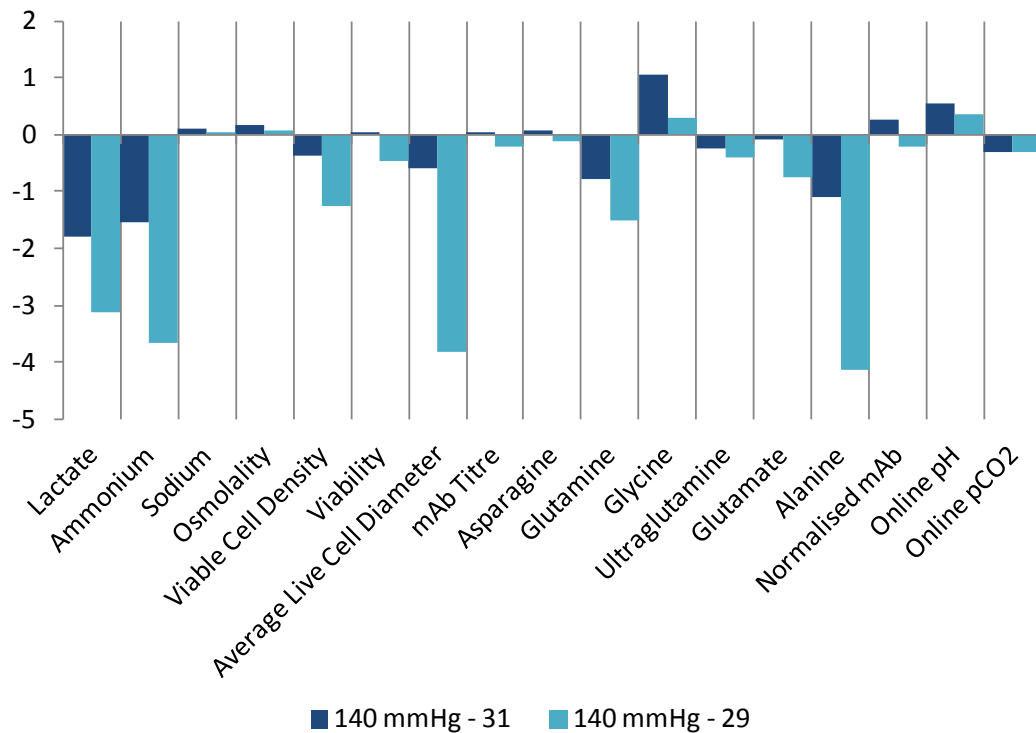
Figure 5.11 - Contribution Plot of PC4 for 140 mmHg (run 31) and 140 mmHg (run 29) Conditions for Nomikos and MacGregor MPCA Model
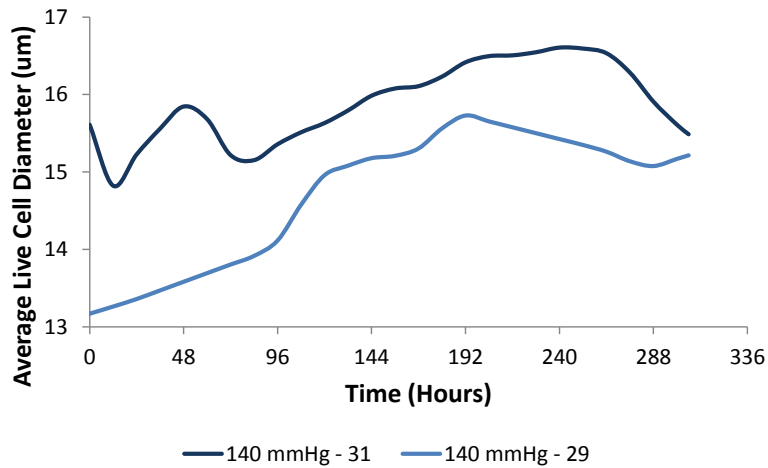
Figure 5.12 - Average Live Cell Diameter for Two 140 mmHg Conditions, Runs 29 and 31
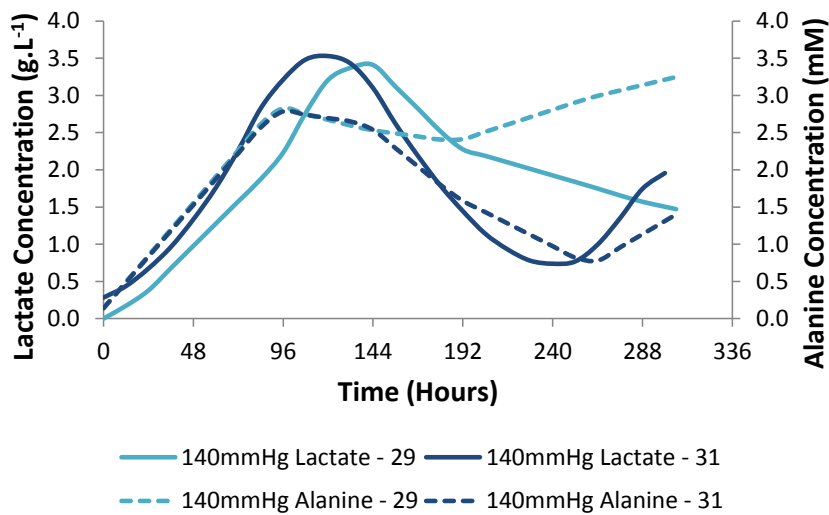
Figure 5.13 - Lactate and Alanine Concentration for Two 140 mmHg Conditions, Runs 29 and 31

PC6 versus PC5

Figure 5.14, the score plot for PC6 versus PC5, indicates a separation of run 32, a 140 mmHg condition from the remaining 140 mmHg conditions. This separation is mainly due to the loadings on PC5. The univariate loadings plot on PC5, Figure 5.15, indicates that the mAb titre, particularly in the early phase has a strong positive loading and that asparagine a negative loading. Other variables such as ammonium and alanine contribute to the differing position of run 32.
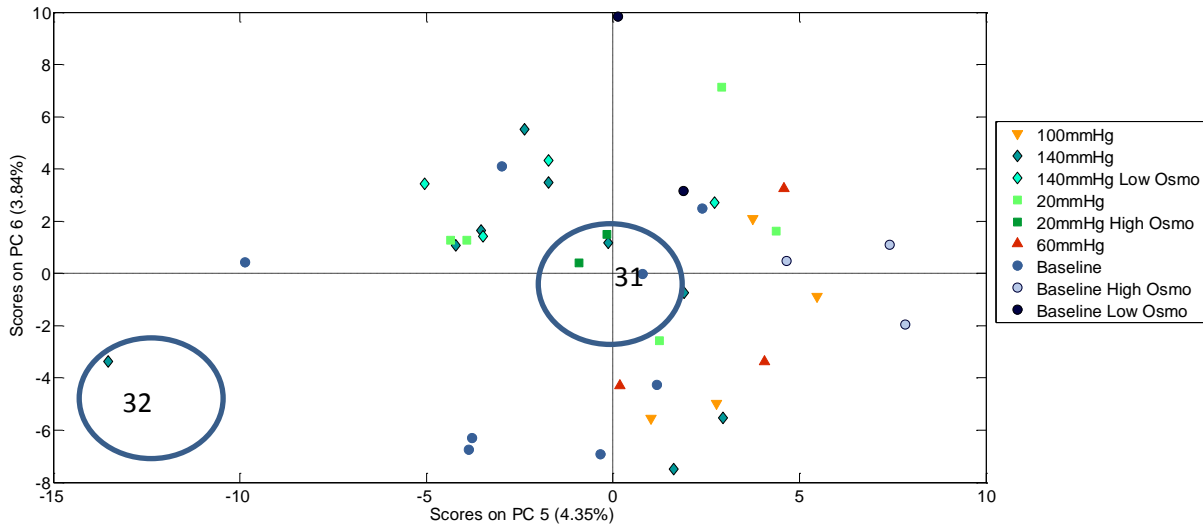
Figure 5.14 - Score Plot for PC6 versus PC5 for Nomikos and MacGregor MPCA Model

Examining the time series plot for mAb for runs 32 and 31, Figure 5.16, indicates lower levels of mAb, along with a delay in production for run 32, compared to the other 140 mmHg conditions. The batch in which run 32 was carried out used a higher passage number (39 for run 32 and 22 for run 31), suggesting that this is the cause of the observed differences. Additionally, there is an increase in Asn usage rate in the run 31 condition during the production of mAb compared to run 32, suggesting a link between Asn usage and mAb production. However, as the amino acids profiles were up-sampled, a firm conclusion cannot be drawn from the Asn usage. Additional work examining the effect of passage number on amino acid usage, with increased sample rates, is required.
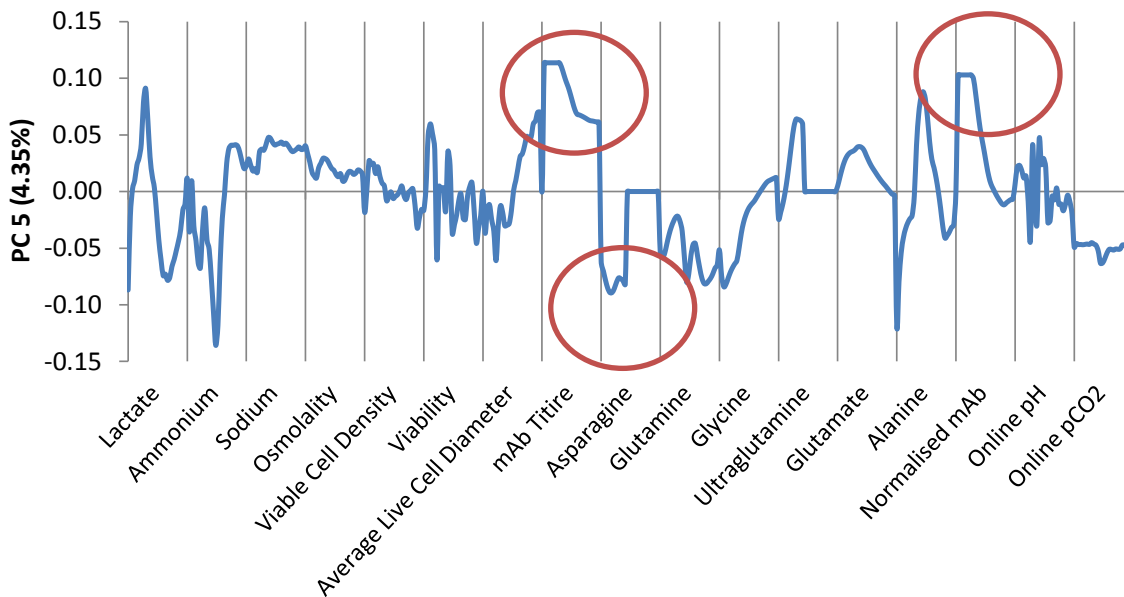
Figure 5.15 - Univariate Loadings Plot for PC 5 for Nomikos and MacGregor MPCA Model

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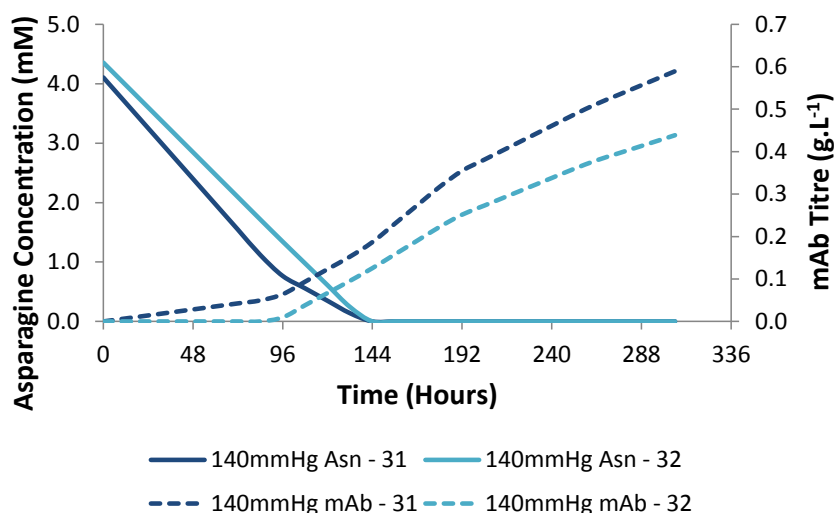
Figure 5.16 - Time Series Plot of mAb Titre and Asn for Runs 31 and 32 (140 mmHg) for Nomikos and MacGregor MPCA Model

Summary on Nomikos and MacGregor MPCA Analysis

Examining the batch data using the Nomikos and MacGregor method has shown clear grouping of runs based on the pCO_2 control and osmolality level. The scores plots summarise the test conditions across the entire cell culture duration and demonstrate clustering due to control conditions. The loadings and contribution plots were used to indicate the cause of the scores groupings for control conditions.

A number of differences in the measured data between STR runs was identified from the PCA model, such as the separation of two 140 mmHg runs from the other 140 mmHg runs, due to differences in cell diameter and alanine and lactate concentrations. The reason for the differences in parameters is currently unknown and it is theorised that it could be due to the passage number used for this run (i.e. clone stability). Further investigation is required to confirm this possible explanation for the differences in parameters, for example by examining the effect of passage number on the cell metabolism. A difference in live cell diameter and asparagine was observed between the baseline and baseline high osmolality conditions. The analysis has suggested that asparagine is a key amino acid relating to mAb production.

5.5.2 MPCA Model - Wold Method

The same data set examined for the Nomikos and MacGregor method was analysed using the Wold unfolding method. The model summary is presented in Table 5.5. Again, six PCs were selected using

the same methodology as for the Nomikos and MacGregor method (see Appendix 8.4.2 for the eigenvalues versus PC plot).

Principal Component	Eigenvalue of Cov(x)	% Variance	% Cumulative Variance
1	7.28	42.80	42.80
2	3.06	18.02	60.82
3	2.39	14.05	74.87
4	1.17	6.88	81.75
5	9.37	5.51	87.26
6	6.87	4.04	91.30

Table 5.5 - Wold MPCA Model Summary

The scores plot for PC2 versus PC1 is shown in Figure 5.17, in which 60.8% of the variance is captured. PC1 predominantly shows how the variables vary over time. The similar pattern for the scores in each control condition indicates that the overall time dependent trends in the data did not change significantly, such as the cell growth and the lactate and ammonium metabolisms. This observation is reflected in the time series data (Chapters 2 and 3), where significant differences in the trend of the overall cell culture parameters were not observed. For example, all conditions have an exponential period of growth, associated with amino acid and metabolite consumption and production, followed by a stationary period and death phase (Figure 2.9 (a)).
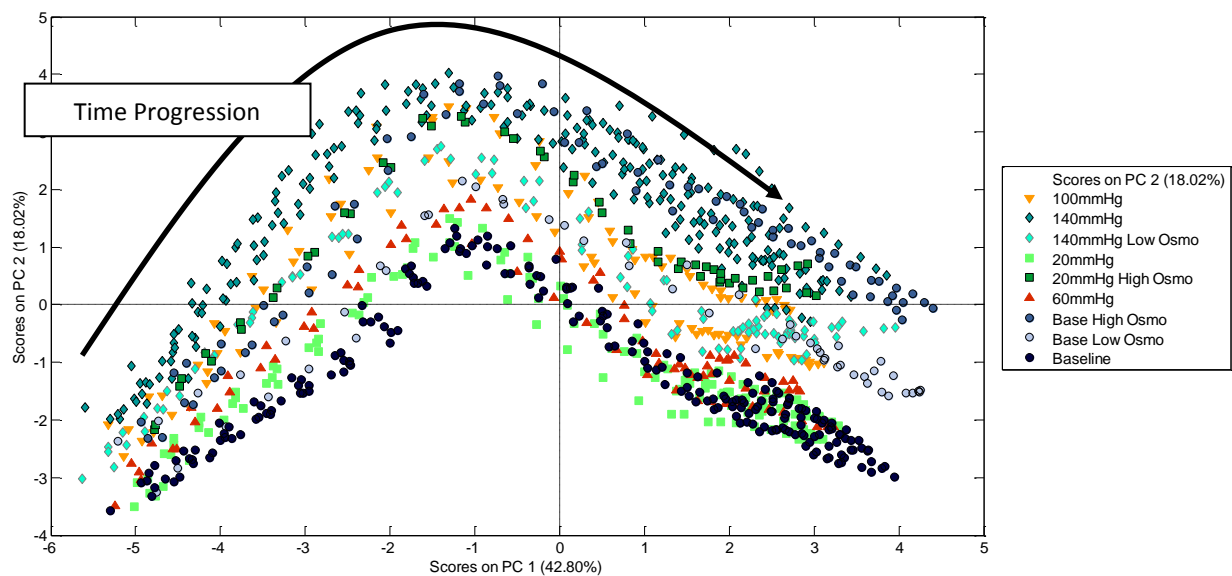
Figure 5.17 - Score Plot of PC2 versus PC1 for Wold MPCA Method

Examining the univariate loadings plot for PC1 and PC2 (Figure 5.18, not to be confused with the contribution plots in the previous sections) indicates that a number of variables contribute to the positioning of the sample scores, i.e. one or a few variables do not dominate. This indicates the complex and interconnected nature of variables within a cell culture process.

The univariate loadings plot for PC1, Figure 5.18, can be used to investigate which variables in the data set contribute to the positioning of the scores in Figure 5.17. On PC1 ammonium, mAb titre, glutamate and glycine have strong positive loadings, which in conjunction with the time series plots are due to an accumulation over time. For example, at the start of a culture, for all conditions, the ammonium concentration is low and hence the scores are located on a negative position for PC1. As the culture progresses the ammonium concentration increases and therefore, the ammonium

variable has a greater influence on the location of the scores on the PC, resulting in a more positive location on PC1.

Cell viability, asparagine and ultraglutamine all have a larger absolute negative loading, illustrated in Figure 5.18, and are shown to be consumed during the culture in the time series plots (Figure 2.12).
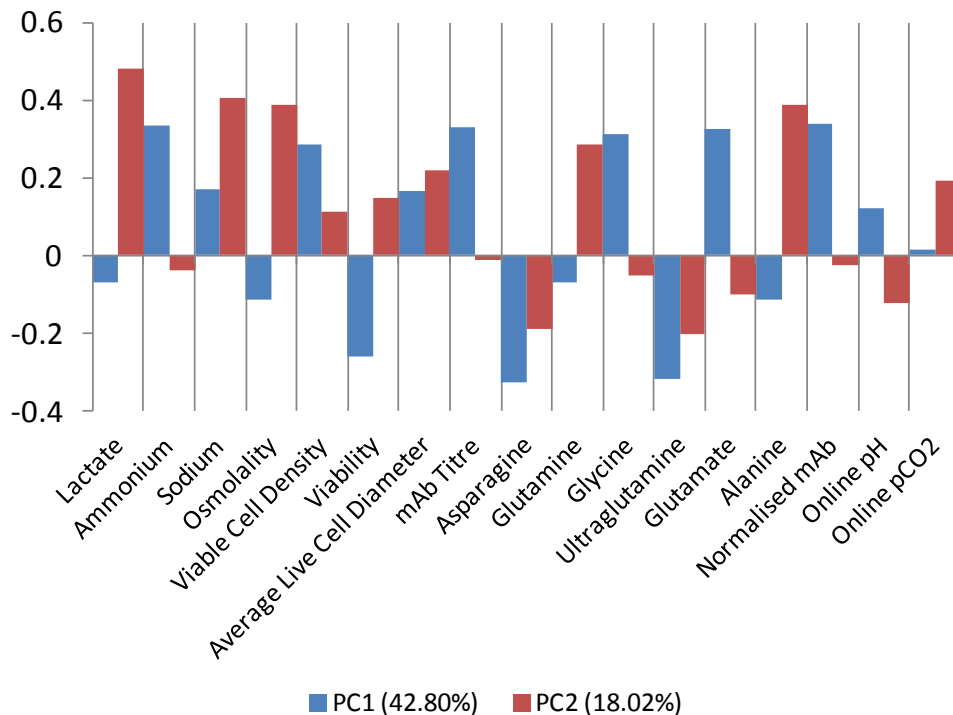
Figure 5.18 - Univariate Loadings Plot of PC1 and PC2 for Wold MPCA Method

The univariate loadings plot for PC2 gives an indication of which variables are responsible for the separation of runs based on the control conditions, and include the levels of lactate, osmolality and alanine, as evident from loadings of a large absolute magnitude. Examining only the pCO_2 control levels, excluding those where osmolality was adjusted (i.e. baseline high and low osmolality, 20 mmHg high osmolality and 140 low osmolality); there is a clear separation of control levels based on pCO_2 level, shown in Figure 5.19. The plot shows the separation of the exponential and stationary phases as indicated by two distinct operating regions where the cell culture parameters change. This suggests that it may be beneficial to create a model for the exponential and stationary phases separately, to explore the underlying behaviour in more detail.
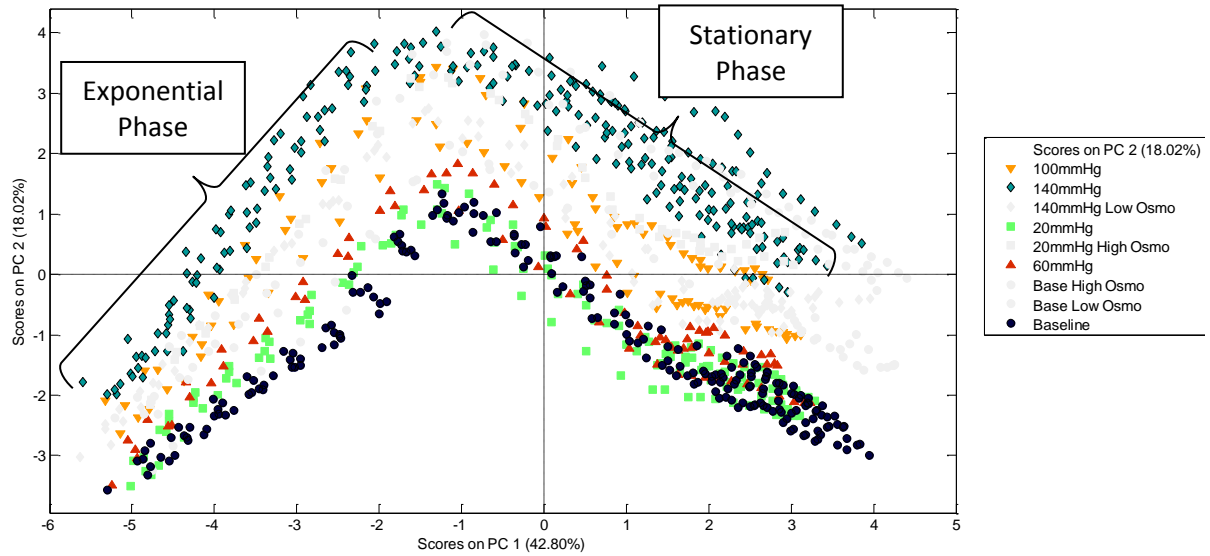
Figure 5.19 - Score Plot of PC2 versus PC1 for Wold MPCA with Osmolality Adjusted Levels Greyed Out

Combing the loadings and scores plots, in the form of the biplot, gives a visual representation of the cause of the score positioning based on the loadings, shown in Figure 5.20 for PC1 versus PC2. The biplot shows that Asn and Glx (glutamine source) have a stronger impact on the process during the exponential phase. This is due to the location of Asn and Glx in the bottom left of the plot, where the sample scores for the exponential phase lie. This observation aligns with the theory that glutamine and asparagine consumption is strongly linked to the cell growth, as discussed in Chapters 2 and 3. Figure 5.20 also indicates that lactate and alanine are shown to have larger absolute loadings towards the mid exponential growth point (about 96 hours) as reflected in the time associated series plot (Figure 2.9 (b) and (c)).
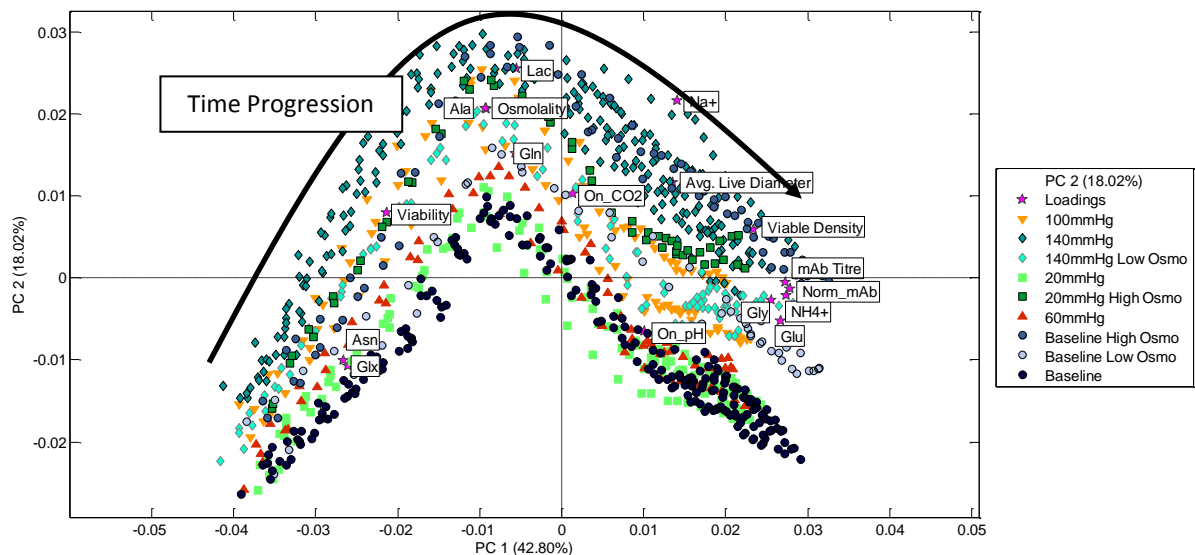
Figure 5.20 - Biplot of PC2 versus PC1 for Wold MPCA Method

PC4 versus PC3

The biplot for PC4 versus PC3, Figure 5.21, shows that the grouping of the conditions is due to a combination of parameters relating to the control conditions. However, this grouping is not as evident as seen in PC2 versus PC1 (Figure 5.20). This is likely due to the dynamic trends in the data and the fact that the cell culture can be defined in two separate stages, with differing responses; the exponential phase and stationary/death phase. This suggests that for analysis using the Wold method, a split between the phases for data analysis would be beneficial.
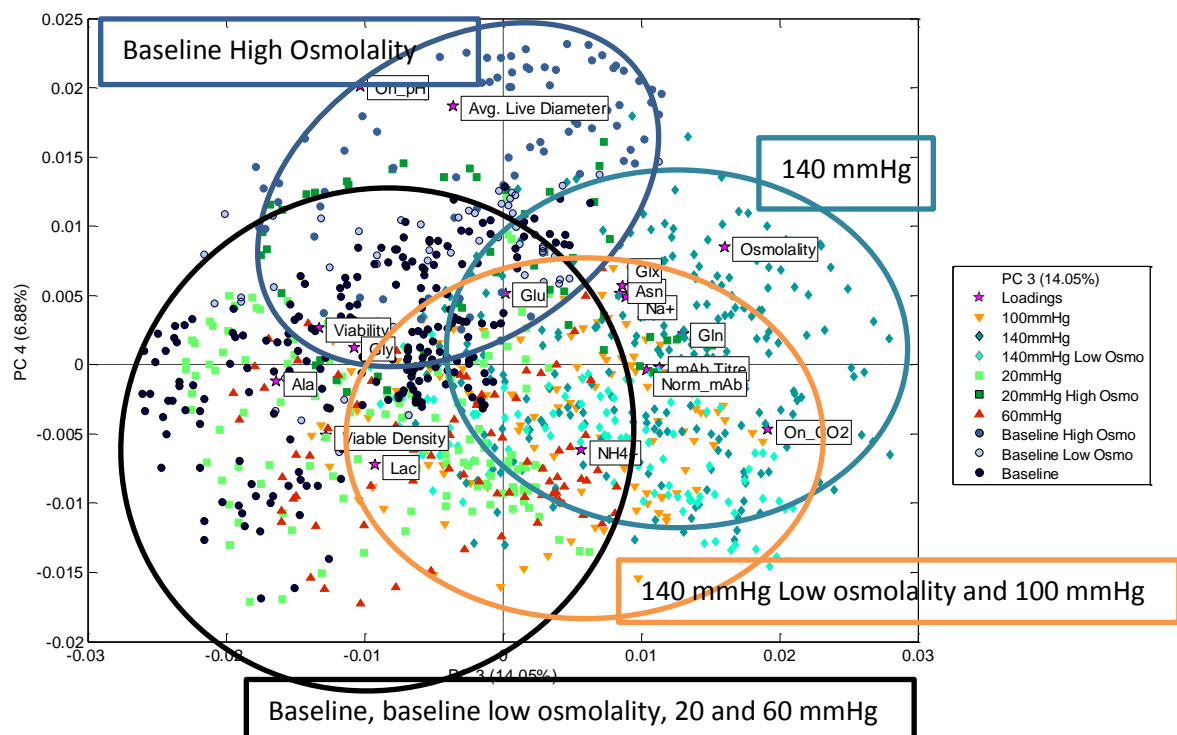
Figure 5.21 - Biplot of PC4 versus PC3 for Wold MPCA

An example of the grouping on PC4 versus PC3 is shown for the 140 mmHg conditions, which are located on the right of the plot (a more positive loading on PC3). The biplot, Figure 5.21, indicates that this grouping is mainly due to osmolality, alanine, lactate and the viable cell density, based on the positioning of samples scores relative to the variable loading. The conditions with a lower osmolality, such as the baseline, and 20 mmHg conditions are grouped in the left hand side of the plot. These groupings summarise the differences in the control conditions, as confirmed by the time series plots discussed in Chapters 2 and 3. A separation of the baseline high osmolality conditions is noted on PC4, in the northern section of the plot, this is due to the loading from the average live cell diameter. As discussed in Chapter 3, the increased osmolality in the baseline condition resulted in an increase in the cell diameter, indicating that the biplot, Figure 5.21, supports this observation. However, as mentioned previously, the groupings on the PC4 versus PC3 are unconvincing, particularly when compared to PC2 versus PC1.

As with PC4 versus PC3, PC6 versus PC5 did not demonstrate robust grouping of conditions, likely due to the two cell phases. However, a separation of the 20 mmHg high osmolality condition was observed, as shown in Figure 5.22. The biplot indicates that this separation is mainly due to the combination of sodium, osmolality, average live cell diameter, glutamine and pCO_2. The high osmolality condition was adjusted to an increased osmolality using sodium chloride, which is likely the cause of the separation of the condition, as it had a differing profile to the 20 mmHg condition. The plots summarise the difference in the parameters for the 20 mmHg high osmolality conditions compared to the other control conditions. The PC combination of 6 and 5 does not show any further grouping based on the control conditions, with a number of condition scores overlapping.
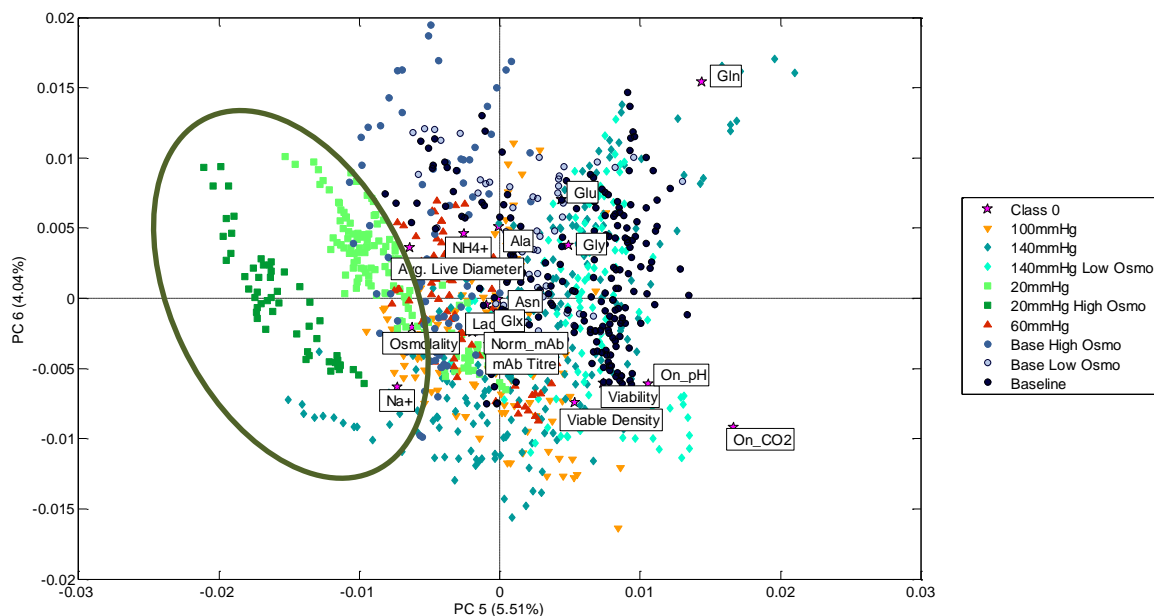
Figure 5.22 - Biplot of PC6 versus PC5 for Wold MPCA

Variable Correlations

As shown on the biplots (Figure 5.20, Figure 5.21 and Figure 5.22), the loadings represent a variable in the cell culture. Therefore, the bivariate loadings plots are useful for highlighting correlations between variables. Variable loadings close to each other (i.e. an angle close to zero from the centre of the PC axis) are positively correlated and variables in opposite quadrants are negatively correlated (i.e. angle of 180). For the correlations to be valid, the relationship needs to be evident in all PC combinations. As shown in Figure 5.23, ultraglutamine and asparagine and lactate and osmolality are positively correlated as they lie next to each other for both PC1 versus PC2 and PC3 versus PC4 plots (as well as other PCs which are not shown). These correlations are confirmed and were discussed in

detail in Chapters 2 and 3, giving evidence to support the observations. A negative correlation between Glx/Asn and viable cell density is present, which supports the link between Glx and Asn depletion and the shift to stationary growth, as discussed in Chapters 2 and 3.
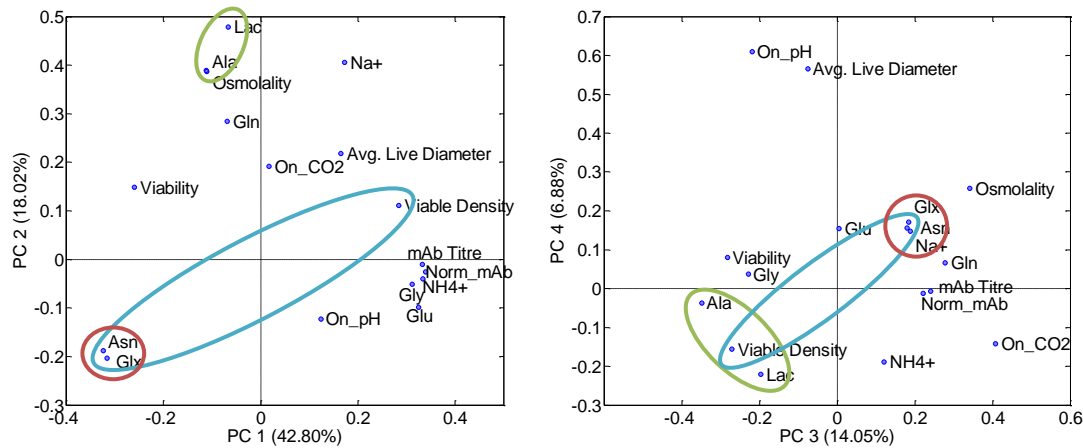
Figure 5.23 - Loadings Plot for Wold MPCA of PC1 versus PC2 and PC3 versus PC4 Indicating Correlated Variables

Summary on Wold MPCA Analysis

The Wold unfolding method demonstrated a grouping of conditions based on pCO_2 control and osmolality level, due to a number of variables, mainly on PC1 and PC2. Analysis of the scores and loadings for PC2 versus PC1 indicated how the PCA plots can be used to interrogate a large data set, highlighting which variables caused a grouping of conditions. In conjunction with the time series plots, the differences in the variables in the data set, relating to control conditions could be summarised and explained. The results gave statistical support to the conclusions drawn in Chapters 3 and 4. Furthermore, from examination of the bivariate loadings plots, an indication of correlations between variables, such as the correlation between Glx/Asn and cell growth was observed and aligned with the discussion in Chapters 2 and 3.

Grouping of control conditions was only strongly evident on PC1 and PC2. Higher PCs did not demonstrate significant grouping, despite explaining relatively significant variation in the data, such as 15% for PC3. It is hypothesised that this was due to the presence of two separate stages in the culture, growth and stationary/death phases, which resulted in differing responses. Therefore, a split of the data and re-analysis using the Wold method would be beneficial.

5.5.3 Data Split Exponential and Stationary

The process data was split according to the exponential and stationary phases of growth and a PCA model was created separately for each phase. The rationale for this split was firstly to enable the inclusion of product quality data (charge variants), which could not be included for the initial model discussed in Sections 5.5.1 and 5.5.2, as samples prior to 144 hours contained concentrations that were too low for analysis. Secondly, the exponential and stationary growth can be considered as separate phases and as discussed previously, the separation of the phases may yield more information specific of the phase as it will not be masked by the other phases.

Acronym	Included Variable
Ala	Alanine
Asn	Asparagine
Avg. Live Diameter	Average Live Cell Diameter
CV_Acidic	Acidic charge variant
CV_Basic	Basic charge variants
CV_Target	Target mAb (charge variant)
Gln	Glutamine
Glu	Glutamate
Glx	Ultraglutamine
Gly	Glycine
Lac	Lactate
mAb Titre	mAb Titre
Na+	Sodium
NH4+	Ammonium
Norm mAb	Normalised mAb Titre
Osmolality	Osmolality
pCO$_2$_On	Online pCO$_2$
pH_On	Online pH
Viability	Viability
Viable Density	Viable Cell Density

Table 5.6 - Included Variables for Exponential and Stationary Phase for Wold MPCA Model

The variables included in the model are listed in Table 5.6. The exponential phase included data from time 0 to 132 hours and the stationary phase included the data from 144 hours till the harvest point, 306 hours.

5.5.4 Exponential Phase Analysis

The summary of the model used is shown in Table 5.7. Six components were maintained (see Appendix 8.4.3 for plots). As discussed previously for data mining purposes, the selection of a higher number of PCs is not detrimental to the analysis.

Principal Component	Eigenvalue of Cov(x)	% Variance	% Cumulative Variance
1	9.17	53.96	53.96
2	2.82	16.57	70.53
3	1.31	7.71	78.24
4	1.08	6.37	84.61
5	8.21E-1	4.83	89.44
6	5.81E-1	3.42	92.86

Table 5.7 - Exponential Phase MPCA Model Summary

Examining the Q residuals versus Hotelling T^2 contribution, Figure 5.24, indicates the presence of a sample outlier. The outlier is due the initial time point for Batch 4 STR 4, which is a 140 mmHg condition. Examining the overall Q residuals and T^2 contributions for this sample, Figure 5.25, shows that the position of the outlier is due mainly to the contribution of cell viability.
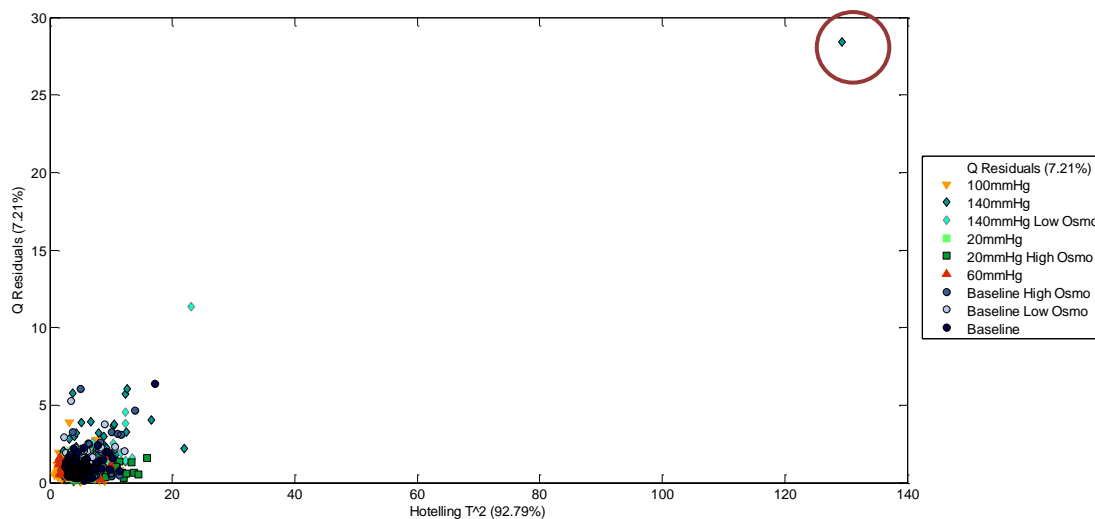
Figure 5.24 - Q Residuals versus Hotelling T^2 plot for Exponential Phase Wold MPCA, Indicating the Presence of an Outlying Sample

Inspection of the cell viability value for this sample indicates a lower than expected value (>95%) of 82.4%. The consequent viability points (i.e. 12 to 96 hours) indicates that this is most likely due to an error on the analytics when determining viability. Therefore, a value of 99% was used to replace it for analysis, which was equivalent to the values of other batches at this time point. This demonstrates how the model can be used to examine for data outlier identification.
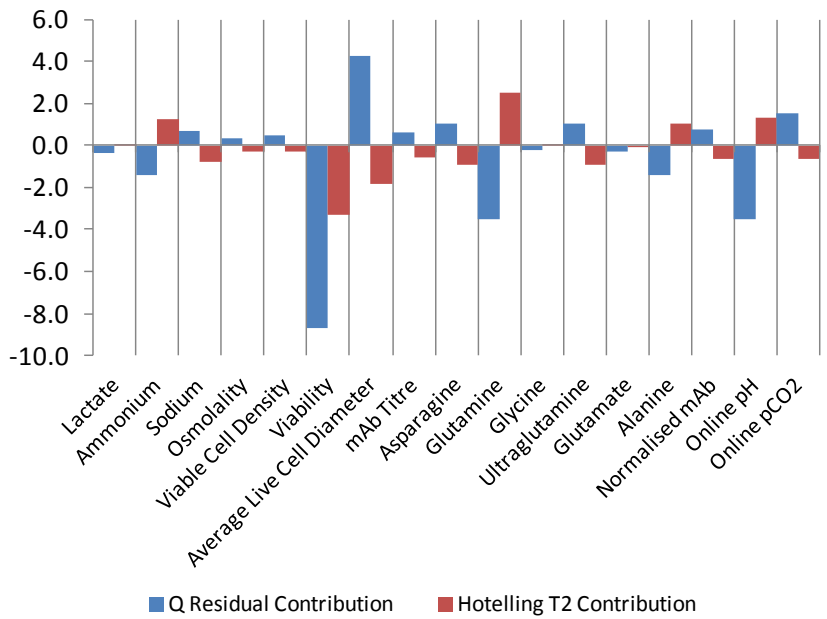
Figure 5.25 - Univariate Q Residuals versus Hotelling T2 plot for Exponential Phase Wold MPCA

PC2 versus PC1

As with the complete data set analysis (Section 5.5.2), PC1 represents the variation over time. Therefore, for this analysis case, the PC1 versus PC2 biplot (Figure 5.26) can be used to indicate how the variables change relative to time. For example, Asn and Glx located in the left of the plot are consumed and parameters located in the right, such as alanine, lactate and ammonium increase with time. Other parameters such as pCO_2, pH and osmolality do not change significantly over time, due to the small loadings on PC1 (i.e. close to zero on the x-axis).
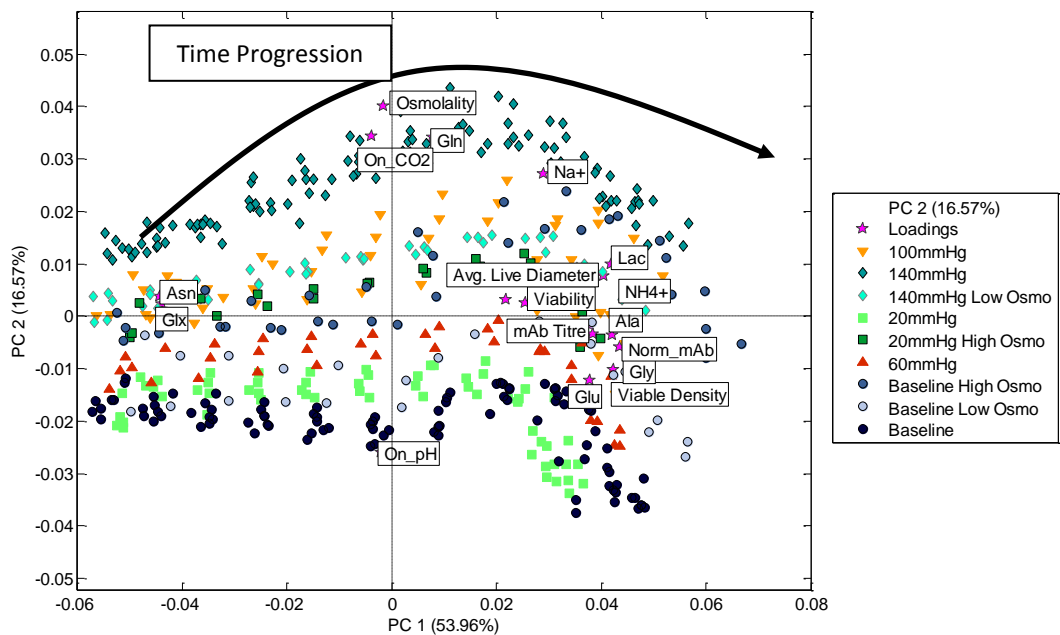
Figure 5.26 - Biplot of PC2 versus PC1 for Exponential Phase Wold MPCA Method

Figure 5.26 shows that only a few variables have a strong loading on PC2, which is the main cause of the separation in the grouping of control conditions. The variables with significant loadings on PC2 are pH, pCO_2, osmolality, sodium and glutamine. The plot indicates that during the exponential growth phase, mainly the osmolality and pCO_2 vary between the control conditions, with little difference between other parameters. The time series plots in Chapters 2 and 3 indicated little difference was between control conditions during the exponential phase, which is also evident in Figure 5.26.

Although pH and glutamine are shown to vary between conditions on PC2, it is unlikely that the differences were significant. During the cell culture, pH was controlled tightly within a ± 0.025 dead-band and the level of glutamine measured was below 0.5 mM, which is the lower limit of detection for the assay. The reason for the strong loading is likely due to the fact that higher pCO_2 control conditions plot at the lower limit of the pH deadband, whilst lower pH condition plot at the higher end of the pH deadband. This indicates that caution is required when drawing conclusions from the model and inspection of the original data set is required.

PC4 versus PC3 and PC5 versus PC6

Examination of higher PC combinations (PC3 to PC6) did not indicate strong groupings based on control conditions. This is likely due the fact that during exponential growth phase, there is little difference in the cell culture performance and hence the measured variables. This is likely the reason that little difference was observed in the previous section when using the Wold method, Section 5.5.2. A grouping of the higher osmolality conditions is observed in Figure 5.27. As discussed in the previous section (Section 5.5.2) this is due to an increase in an average live cell diameter when the osmolality was increased in these conditions.
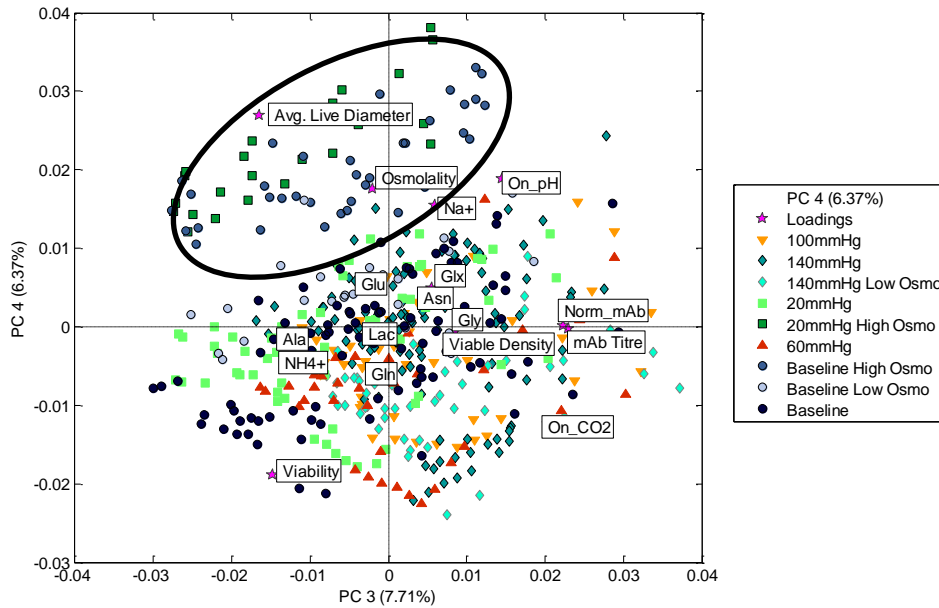
Figure 5.27 - Biplot of PC4 versus PC3 for Exponential Phase Wold MPCA Method

Variable Loadings

The bivariate loadings plot, Figure 5.28, shows that ultraglutamine and asparagine are positively correlated and are, in turn, negatively correlated with lactate and ammonium. Ultraglutamine and asparagine are consumed for cell growth. As cells grow, they produce lactate and ammonium, which are by-products of growth related metabolism. A negative correlation is shown between osmolality and viable cell growth and pCO_2, which was an observation made in Chapters 2 and 3.
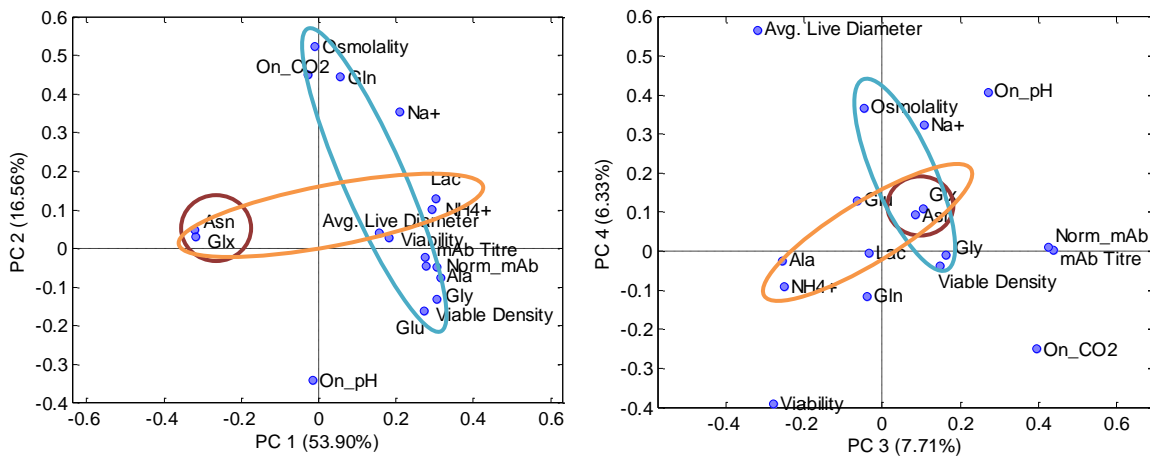
Figure 5.28 - Loadings Plot for Exponential Phase Wold MPCA of (a) PC2 versus PC1 and (b) PC4 versus PC2, Indicating Correlated Variables

Exponential Phase Analysis Summary

The exponential phase model indicated that there was grouping of the control conditions, however, fewer variables contributed to the positioning than for the stationary phase. The main differences indicated between sample scores were in pCO_2, osmolality, sodium, glutamine and online pH.

The small loadings for a number of other parameters, such as lactate and ammonium show that there was little difference between these parameters across the runs (i.e. control conditions). The small difference between the growth-related metabolism by-products demonstrates that cell growth is the dominating cell activity in the early stages of the culture.

The scores and loadings plots showed, as with previous models, that the process of increasing the osmolality, with the addition of sodium salt resulted in a change grouping of control conditions, mainly due to an increase in cell diameter.

The summary of the model developed for the stationary phase using the Wold method is shown in Figure 5.8. Six components were retained from an examination of the eigenvalue plot when a knee point (i.e. a 'bend' in the plot) occurred at 6 PCs (see Appendix 8.4.4 for plots).

Principal Component	Eigenvalue of Cov(x)	% Variance	% Cumulative Variance
1	6.23	34.59	34.59
2	4.32	24.00	58.59
3	2.38	13.21	71.79
4	1.92	10.68	82.47
5	1.12	6.24	88.71
6	4.28E-1	2.38	91.09

Table 5.8 - Stationary Phase MPCA Model Summary

Examination of the Q residual and Hotelling T^2 plot (Figure 5.29) indicated that there were no significantly deviating batches, as the majority of the scores lie on top of each other.
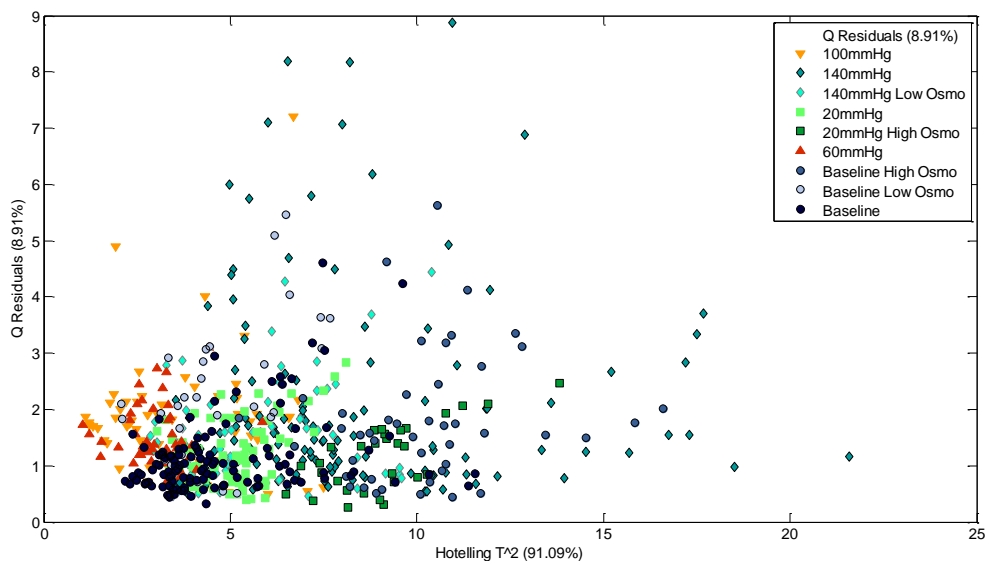
Figure 5.29 - Q Residuals versus Hotelling T2 Plot for Stationary Phase Wold MPCA Model

PC2 versus PC1

The biplot of PC1 versus PC2, Figure 5.30, explains 58.6% of the variability in the data set. PC1 represents the change in parameters over time. Parameters located to the left of the plot are associated with a decrease in value, such as alanine, lactate, cell viability and target mAb (CV_Target), when examined with the time series plots. Variables located in the right of the plot (i.e. positive loading on PC1) are associated with an increase with time, such as ammonium, acidic charge variants and mAb titre. Variables close to 0 for PC1, such as glycine, online CO_2 and osmolality had little change in across time during the stationary phase.
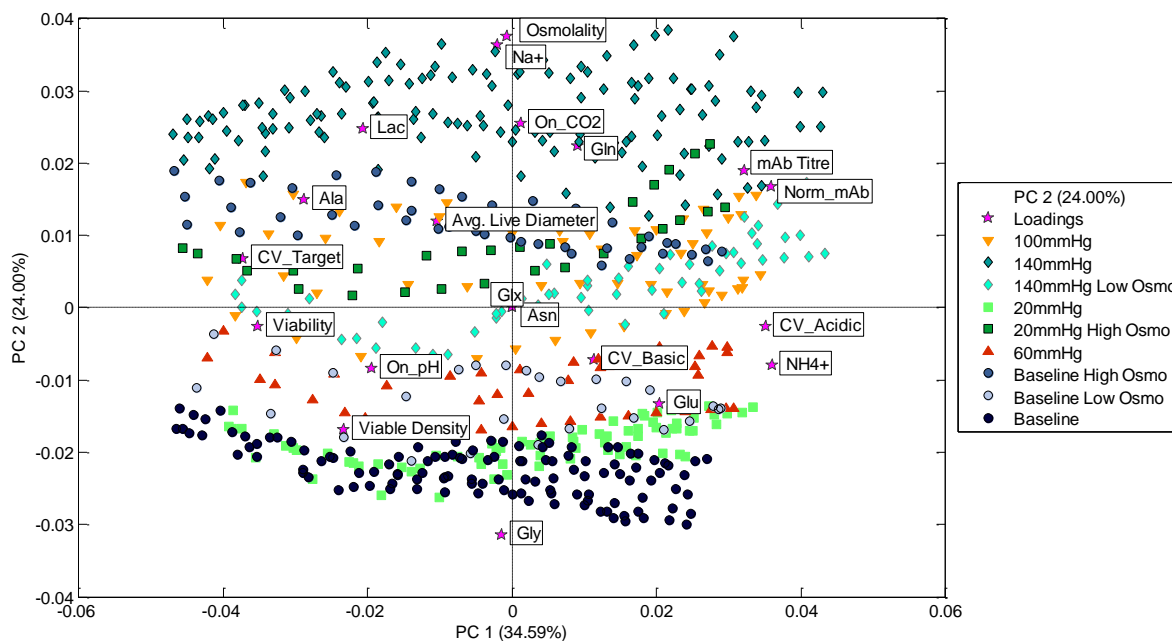
Figure 5.30 - Biplot of PC1 versus PC2 for Stationary Phase Wold MPCA

There is little difference relating to the pCO_2 control and osmolality levels on PC1, indicating that there was little difference between the overall trends across the levels examined, i.e. the time related structure of the data. For example, there was an accumulation of ammonium and consumption of lactate in all conditions, such as those shown in Figure 2.9**.**

Grouping of control conditions across PC2 can be observed in Figure 5.30, relating to differences in a number of parameters, mainly glycine, osmolality, sodium, lactate, viable cell density and pCO_2. An increasingly positive loading on PC2 is associated with increased pCO_2 and osmolality levels. The plot shows that there are clear differences in a number of variables due to pCO_2 control and osmolality level during the stationary phase.

The baseline conditions and low pCO_2 levels are associated with higher glycine concentrations and viable cell density shown by the closer proximity to these variables on PC2 and examination of the time series plots (i.e. the combination of variable value and the variable loading results in a more negative score). Condition scores with a positive loading on PC2 are associated with higher lactate, alanine, mAb titre, pCO_2 and osmolality/sodium. Parameters located closer to the centre of the axis (i.e. 0) indicate that there was little difference between variables relating to the control levels, such as viability, ammonium and Asn/Glx.

An overlap of the baseline high osmolality condition is noted with the 100 mmHg condition in Figure 5.30, suggesting similar performance, which is likely due to the increase in osmolality. This highlights the importance of osmolality to cell culture performance.

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PC4 versus PC3

A grouping of scores based on control conditions is seen in Figure 5.31, the biplot for PC4 versus PC3. A separation of control conditions on PC3 is observed due to a number of variable loadings. The grouping of conditions on the west of the plot (negative on PC3) is influenced strongly by the basic charge variants. This is reflected in the univariate loading plot for PC3, Figure 5.32. Examining the time series plot indicates that this is due to a higher level of basic CVs in the 20mmHg conditions and conditions located further from the basic CV loading have lower levels of this variant. Conditions located on the east of the plot have a lower level of basic variants, with the baseline and 140 mmHg conditions located furthest to the west.
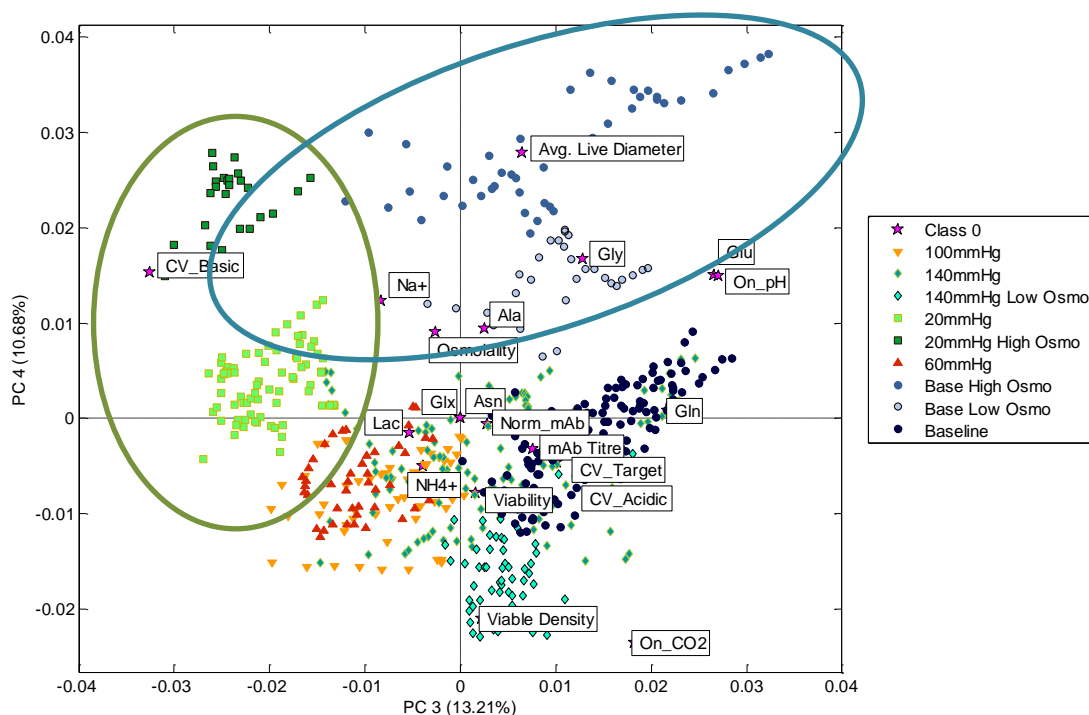
Figure 5.31 - Biplot of PC4 versus PC3 for Stationary Phase Wold MPCA

On PC4, the high osmolality conditions for the baseline and 20 mmHg conditions are located on a positive loading. As indicated in Figure 5.32, the univariate loadings for PC4, the score locations are mainly influenced by the average live cell diameter, viable cell density and pCO_2. Examining the time series plots for these conditions indicates that this is due to the combination of the reduced cell growth and increase in cell size, which occurred at a lower pCO_2 level (as discussed in sections 5.5.1 and 5.5.2). This supports the conclusion arrived in Chapter 3 that increased osmolality resulted in reduced viable cell density and increased average live diameter.
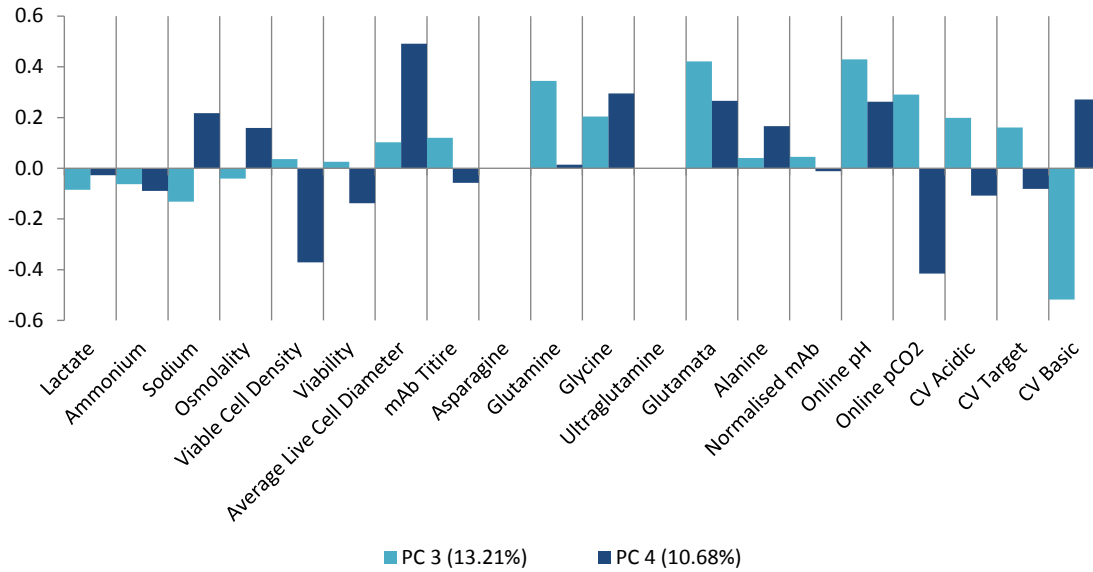
Figure 5.32 - Univariate Loadings Plot of PC3 and PC4 for Stationary Phase model

Variable Loadings

Examining the bivariate loadings plot for the stationary phase,

Figure 5.33, indicates a number of correlated variables. Figure 5.34 indicates that the basic charge variants and pCO_2 level are negatively correlated, as was discussed in Chapters 2 and 3, giving statistical backing to the observation. Alanine and ammonium are shown to be strongly negatively correlated, which supported the observation made in Chapters 2 and 3 that the metabolism of alanine and ammonium is strongly linked.
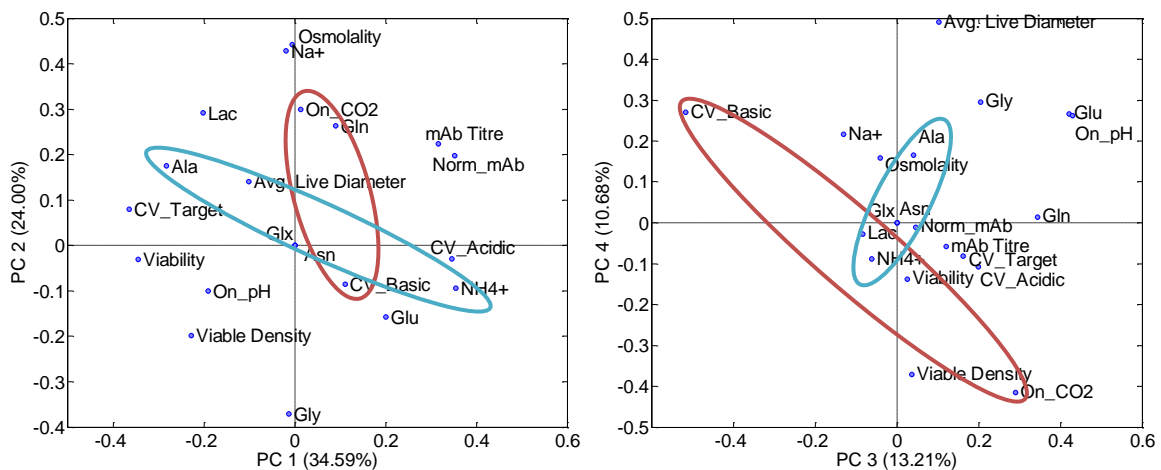
Figure 5.33 - Loadings Plot for Stationary Phase Wold MPCA of (a) PC2 versus PC1 and (b) PC3 versus PC2, Indicating Correlated Variables

The PCA model for the stationary phase has indicated groupings based on the control conditions. The subsequent scores and loadings plots have been used to interoperate the grouping of conditions and to summarise the effect of pCO_2 control and osmolality level on the process. The biplots indicated that the main differences between control conditions were due to pCO_2, osmolality, glycine, lactate and alanine. However, a number of variables contributed to the positioning of the samples, indicating the multivariate nature of the cell metabolism.

The model has been used to statistically support a number of observations made in the previous chapters, such as that increasing pCO_2 resulted in lowered basic variants and that Glx/Asn consumption is linked to cell growth. The observation of ammonium and alanine being strongly correlated was supported. This finding supports literature, where the catabolism of alanine has been linked to the increase in ammonium. However, process knowledge is required when interoperating the plots, as alanine consumption is not the sole source of the ammonium production and a number of other factors contribute to the level of ammonium in the culture. This is shown in the baseline conditions, where a reduction in ammonium is noted despite higher levels of alanine, which is likely due to a lower TCA cycle activity (as discussed in Chapters 2 and 3).

The inclusion of the product quality attributes (charge variants), indicate a number of correlations to other variables, such as pCO_2 level, basic CV, target CV and ammonium. Ammonium has been discussed in the literature as being detrimental to cell culture processes, however, further investigation is required to determine if this is the cause of the decrease in product quality at a pCO_2 control level of 20 to 100 mmHg. Further knowledge was not extracted in the current study relating to the cause of improved product quality at 140 mmHg compared to pCO_2 control from 20 to 100 mmHg. Therefore, the effect of pCO_2 on product quality is likely due to an unmeasured variable(s), such as the pH_i as discussed in Chapters 2 and 3.

5.6 PCA Conclusions

PCA representation demonstrated grouping of pCO_2 control and osmolality levels due to differences in process variables. The analysis has given statistical justification to observations made from the time series plots discussed in Chapters 2 and 3. The analysis indicated that pCO_2 control did result in significant differences in the process, relating to cell growth, metabolism and mAb production.

New knowledge relating to the increased cell diameter in conditions where osmolality was adjusted manually has been obtained. Further investigation is required to determine the cause of the increased cell size. This may be due to the cells reaching equilibrium in osmolality (between the

external and internal osmolality), leading to an influx of ions into the cell. However, measurement of the cell contents is required to confirm this hypothesis.

The scores and loadings plots provided statistical backing to the study findings that consumption of glutamine and asparagine are linked to cell growth and the switch between growth phases. However, a DoE study into the effects of glutamine and asparagine on cell growth and metabolism is recommended to confirm this finding.

The PCA work highlighted differences between two of the 140 mmHg runs, in which there was lower consumption of lactate and alanine and lower levels of ammonium production compared to other 140 mmHg runs. Currently, the cause of this finding is unknown and it is hypothesised that it is likely due to clone stability; however, further investigation is required. The data supports the theory that higher levels of lactate leads to a lower consumption rate of alanine and a lower production rate of ammonium.

Further knowledge from the interrogation of the models could not be achieved during the study due to a number of key variables not being measured, such as the pH_i and internal cell metabolism. Overall, the PCA results statistically confirmed that pCO_2 control leads to operational differences in the cell culture performance.

Careful interpretation of PCA models as well as the data included in the model during analysis is required. Correlations indicated in the PCA model does not prove causation or a true correlation, as the model is dependent on the data used, as with any modelling technique. The results of the PCA should not be final, but should lead to further investigation to confirm or disprove observations drawn from the model. For example, during the model development the amino acid hydroxyproline was included and the variable had a large loading on the model. However, this was due to the fact that hydroxyproline was measured at very low concentrations and inaccuracies from the detection below the lower limits of the assay falsely indicated that it varied significantly. Therefore, using process knowledge it was removed as it was known not to be a true variation in the runs.

PCA cannot be used on its own to gain further understanding or for development of a process. It is required alongside DoE and modelling tools, as highlighted by Huang *et al* (2009), for process understanding and development. Additionally, the correct parameters need to be measured and included in the model.

MVDA techniques such as PCA cannot be considered as a 'one-size fits all' technique. As with any modelling technique, clear analysis aims, process knowledge and sufficient and relevant data are required. Rather than being a method to extract unknown information, PCA should be considered as

a tool to generate models for defining specific processes, leading to further investigations via DoE, i.e. PCA on its own, without prior process knowledge or further analysis tools is ineffective.

The steps in PCA are an iterative process involving data pre-processing, dimensionality reduction and interpretation of results. These steps can take a significant amount of time with data pre-processing being the most substantial. Data pre-processing steps transform the data into a suitable form for principal component analysis, which involves data cleaning, normalisation and missing data handling. The main challenge for data pre-processing is the varied data types and sample rates and the presence of missing and anomalous data. Therefore, the examination of historical data sets requires a significant amount of time to interpret the input data. An added complication for PCA is that a value is required for each variable/batch at each data point analysed. Therefore, certain data may not be suitable for analysis due to missing/anomalous data points or due to the type of data, i.e. continuous or categorical. Data sets with these data forms either require data treatment assumptions, which can potentially lead to incorrect interpretations of the results if improperly applied, or result in the data set not being included in the analysis.

Despite the significant amount of time required for data pre-processing, this process is generally shorter than univariate analysis of the data, which in large data sets is extremely difficult without missing data trends.

6 Research Conclusions and Recommendations for Future Work

6.1 Influence of pCO₂ Control and Osmolality Level on a Mammalian Cell Culture

Mammalian cell lines, like Chinese hamster ovary cells, are used in the biopharmaceutical industry due to their ability to carry out post-translational modifications (PTMs) of proteins required for human use. Mammalian cells, however, suffer from slow growth and high nutrient consumption rates and accumulation of toxic metabolites. These factors can result in low cell growth rates, productivity and product quality. Therefore, the monitoring and control of parameters affecting the cell growth is essential to improve productivity whilst maintaining/improving product quality. Much effort has been conducted in the literature, examining the biological, chemical, environmental and mechanical parameters which effect cell culture systems.

One parameter, of which its effect on cell culture is poorly understood, is the level of carbon dioxide partial pressure (pCO_2). Carbon dioxide levels vary with scale, with a build-up to high levels (>100 mmHg) at the larger scale and low levels at the small scale. Carbon dioxide has been shown in the literature to affect the productivity and product quality of cell culture processes; however, there is a lack of literature on the effect of pCO_2. There is a lack of consideration for the control or effect of pCO_2 on cell culture, especially during quality by design and scalability studies. If pCO_2 control has the potential to effect/improve cell culture performance, then the use at production scale would be beneficial. Additionally, for continuous operation, such as perfusion processes, the selection of a pCO_2 level will be required. Therefore, this research was undertaken to assess the effect of pCO_2 on a cell culture process, i.e. if it is a critical process parameter.

The aim of the research was to determine if pCO_2 control level affected a CHO cell culture process. This was determined by assessing the effect of pCO_2 control level on productivity, product quality and cell metabolism in comparison to a traditional process (baseline condition) where pCO_2 level was varied to control pH level.

The study results demonstrated that pCO_2 control level affected the productivity, product quality and cell metabolism of a CHO cell culture. Increasing pCO_2 control level resulted in an increase in osmolality levels, both having an effect on the cell culture process. Both the increase in pCO_2 and osmolality level resulted in increased productivity compared to the baseline. Operation at low to high pCO_2 (20 to 100 mmHg) gave reduced product quality attributes, demonstrated by charge

variant and N-glycan analysis, compared to the baseline. Operation at elevated pCO_2, 140 mmHg, resulted in equivalent product quality attributes to the baseline, at an elevated productivity level.

The current research results differ from those reported in the literature. However, published literature on the subject is limited and the relevant study has been mainly carried out by one research group (deZengotita *et al,* 1998; deZengotita *et al* 2002; Kimura and Miller, 1996; Kimura and Miller, 1997). It has been noted that unrepresentative culture systems have been used (e.g. T-flask and well plates) in the literature, which lack the monitoring and control capabilities of the production vessels.

Monitoring and control of cell culture processes have substantial effects on performance as well as interacting with the effect of other process parameters. For example, a number of studies made use of T-flasks, which do not involve mixing, whereas production vessels traditionally use mechanically driven impellers. This difference in mixing leads to differences in cell performance and also in the aeration of the culture. This is likely to impact on the pCO_2 studies which essentially assesses the effect of aeration on the culture (i.e. the composition of air, oxygen and carbon dioxide). Also, the majority of literature has been published over 15 years ago, in which time significant advances in processing technologies and techniques, product titres, media and cell lines has occurred.

The general current understanding in industry is that elevated pCO_2 is detrimental to process performance. This conclusion appears to have been determined from observations of correlations between poor performing batches and elevated pCO_2 levels. However, the correlation does not mean causation as the combinations of poor mixing and addition of CO_2 to control pH leads to elevated pCO_2 levels. Therefore, it is more likely that elevated pCO_2 level is the result of poor process performance and not the cause.

The current work has shown that pCO_2 level needs to be considered during process development, including the interacting effect of other process parameters and scale effects on the performance. Further examination of elevated pCO_2 levels at industrial scale reactors is required, to determine if elevated pCO_2 is a result of cause of poor process performance.

6.1.1 *pCO$_2$ Control Impact on Bioprocessing*

There are two main impacts of the conclusions from the pCO_2 control level study, relating to the scale-up and continuous operation (e.g. perfusion) of bioprocesses.

Scale-up

Carbon dioxide concentrations vary with process scale. In industrial scale vessels there is a build-up of pCO_2 due to a combination or process performance (i.e. increased pCO_2 to control pH) and poor mixing/gassing leading to poor removal of CO_2. Therefore, the pCO_2 profiles between the small (e.g. 2L bench top) and production scale (e.g. 5000L) are not comparable. The current work has highlighted that pCO_2 level affects a cell culture process. Therefore, differences in pCO_2 profiles between scales can potentially affect the process performance. During process development and scale-up analysis, investigations into the effect of pCO_2 control level would be beneficial, for improved scalability and comparability between scales.

The assertion of the effect of elevated pCO_2 on cell culture process has been made from a small number of studies, with few examining the effect of different pCO_2 levels. As mentioned previously, the issue of pCO_2 build-up at the large scale is one of process optimisation and performance, highlighting that the factors that leading to pCO_2 build-up, such as low gassing and agitation rates and un-optimal control of the culture (e.g. pH), need to be optimised at small scale and during scale-up.

Towards Continuous Processing

The true benefit of the pCO_2 control level work is for consideration toward continuous manufacturing, such as perfusion processes, which is currently the leading method for the push towards continuous bioprocessing. Generally in perfusion systems, a continuous cell culture is fed continually with fresh media and waste media removed and ran at a fixed cell concentration. The culture time is significantly extended over batch and fed-batch. Perfusion is a form of process intensification, i.e. the scale-down of a process whilst maintaining or increasing process output, resulting in cost and waste benefits.

Perfusion processes operate in pseudo-steady state, meaning that, in general, process parameters are constant, such as pH and pCO_2 level, which are defined by the composition of the medium. For perfusion cultures, a constant pCO_2 level will inherently occur, therefore, a decision is required to specify the level.

Commercially available media generally has a pCO_2 level of 60 mmHg (for the given pH level), such as FortiCHO (Life Technologies). However, this work has demonstrated that pCO_2 control at 60 mmHg leads to reduced product quality. Therefore, for perfusion processes the selection of an optimal pCO_2 control and osmolality level is required and the current work has demonstrated that the chosen levels will impact the process. This is a consideration required during the development of

perfusion media and processes to develop to optimal pCO_2, pH and osmolality blend of the media (along with the amino acid and nutrient requirements).

6.1.2 EngD Project Business Impact for Pall Corporation

There have been a number of business impacts of the project for Pall Corporation. These include data on the use of pCO_2 sensors for Pall bioreactors, increased process knowledge of the cell line, generation of processes for product and process development and the creation of procedures and set-up of process for future work and training.

It would be recommended to allow for the accommodation of pCO_2 sensors into the Pall bioreactor range. Currently, the Pall bioreactors include the Micro-24 MicroReactor system (M24), XRS 20 Bioreactor System and Allegro™ STR 200 Single-Use Stirred Tank Bioreactor, Figure 6.1.

Figure 6.1 - Top Left: XRS 20 Bioreactor System, Bottom Left: Micro-24 MicroReactor System, Right: Allegro STR 200 Single-Use Stirred Tank Bioreactor

The Pall Micro-24 MicroReactor system (M24) is a scale-down tool for bioprocess development. Traditionally, small-scale tools are used in quality by design studies to determine the critical process parameter. Additionally, microbioreactors can be used for clone and media selection. Therefore, to improve the comparability of the device and amount of information that can be generated, the incorporation of a pCO_2 sensor would be recommended. Disposable optical pCO_2 sensors are

commercially available; however, a study would be required to determine the accuracy and usability of the sensor on the M24.

The XRS20 system is a disposable rocker system and it would be beneficial to include a disposable pCO_2 sensor as standard. This would give the product a potential competitive advantage over other systems, with claims that pCO_2 is a critical process parameter and the additional knowledge which can be collected through the sensor.

The Allegro STR 200 is a disposable bioreactor system. As with the XRS the recommendation to allow for the insertion of pCO_2 sensor could give the product a competitive advantage. For this purpose the analysis of the scale-up of the pCO_2 control process is required.

Secondary Outcomes of Research Project

An outcome of the research project was the generation of historical data for the use at Pall for future cell culture runs. For example, the comparison of large scale data to the smaller scale has been used to assess the scalability and the process performance of the vessels, as well as indicating if the process was in 'normal' operation.

The data generated in the bench top scale STRs has been used for process knowledge for further process development. For example, the process and process knowledge was applied whilst developing the cell culture processes for the XRS20 and 200L system.

The set-up of the at-line analytics (Nova Flex autosampler), process monitoring program (Biocommand®, Eppendorf) and the operating procedure for small scale STRs were developed and these have been applied for operation and training of employees at Pall Corporation on the use of the STR and at-line analytics. The at-line analytics allows for automated sampling and operation with the use of Biocommand.

6.1.3 Limitations of the Research

There were a number of limitations in the current research study which would require addressing for future work. In bioprocesses, cell line and process related specifics appear to have a significant impact on the effect of parameters on the cell culture. For this study, a single cell line and process was examined, therefore, cell line and process specifics for the effect of pCO_2 on this process cannot be ruled out.

Additionally, the cause of the effect of pCO_2 on product quality was not determined. It is likely that the change in product quality, seen with pCO_2 control, is due to the effect of carbon dioxide

concentration on the internal pH and cell metabolism. The internal pH and cell metabolism were not measured in the study. There are a number of methods for measuring internal cell pH and metabolism, however, they are time consuming, costly, and require expertise and a large amount of modelling. Ideally, online measurement of the pH_i and internal cell metabolism would be preferred, but currently it is not possible.

The effect of pCO_2 has been examined in a non-steady state process (i.e. a batch process), in which there are numerous possible interacting parameters, which change relative to time. Examples are the increase in osmolality with pCO_2, the change to process (metabolism) intermediates which affects the processes (such as lactate production) and different effects of parameters on the cell culture process during different cell growth phases (as shown in the PCA work). A pseudo steady-state system, such as a perfusion process, may be more ideal system to examine the effect of pCO_2 level, as the effects of other interacting parameters on the process can be reduced.

The cell line instability, along with other parameters, led to batch-to-batch variability. This made comparisons between batches difficult. A baseline in each batch was used to normalise the data from pCO_2 controlled runs against. This process of normalisation, however, would result in the inclusion of the variability of the baseline performance in the normalised data. Therefore, the variability in the baseline conditions performance is required. The variability of the baseline performance was assessed prior to the pCO_2 study; however, this was carried out in one batch before the clone stability issue was highlighted.

As part of the current study the instability in the cell line was highlighted and investigations are currently ongoing into the clone line stability by the R&D Application teams at Pall Corporation. A more detailed understanding of the process and cell metabolism is required, such as modelling of the cell metabolism.

The scale-up performance of pCO_2 control was not assessed. This was due to a lack of availability and substantial cost of a larger scale cell culture run. Theoretically, the scale-up of a pCO_2 controlled process would lead to improved comparability; however, it needs to be confirmed practically.

6.2   Principal Component Analysis

Principal component analysis was applied as an exploratory data analysis tool for cell culture data for increasing process knowledge and to demonstrate the application of PCA to cell culture data. The outcome of the PCA is to increase process understanding through the identification of key

parameters and also the identification of relationships between the variables, in particular, the change in these parameters across the pCO_2 control levels.

PCA was applied with the aim of extracting further understanding from the pCO_2 control and osmolality level data set. A data set of 40 STR runs across seven batches, were carried out to examine the effect of pCO_2 control and osmolality level on a mammalian cell culture process.

The PCA work highlighted that there were differences in process parameters relating to pCO_2 control and osmolality level. The work gave statistical backing to a number of observations made in the pCO_2 and osmolality study through the inspection of time series plots. Statistical backing to observations such as the correlation of L-alanine-L-glutamine (Glx) and asparagine to cell growth was noted.

A number of new observations were made, such as increased cell diameter with increased osmolality conditions and a separation of two 140 mmHg runs (due to a combination of parameters), possibly due to the clone line stability. Further knowledge from the interrogation of modelling results could not be achieved. This is mainly due to a number of key variables not being measured, such as the pH_i and internal cell metabolism. Overall, the PCA results statistically confirmed that pCO_2 control leads to operational differences in the cell culture performance.

PCA (and other MVDA techniques) cannot be applied without clear analysis aims, process knowledge and sufficient and relevant information. Rather than being a method to extract unknown knowledge directly from a process, PCA should be applied to re-examine time series data and lead to further investigation, likely via DoE. PCA on its own is not productive but when used with process knowledge and DoE studies can be a powerful tool.

Results from PCA plots can often be difficult and complex to analyse compared to time series plots. A greater understanding of PCA and the resulting plots is required, than the simpler method of examining time series plots. However, inspection of time series plots alone can lead to the missing of correlation between variables and runs. Therefore, a combination of PCA with examination of time series plots is essential.

The study showed the usefulness of applying PCA to the data to extract further knowledge, and to summarise large amounts of data into single plots describing the entire data set. The entire process of creating the PCA model and the analysis of the model resulted in increased process knowledge. However, the process can be complicated and complex plots may be produced which need interpreting and a level of expertise is required to understand, compared to time series plot analysis. PCA needs to be used alongside modelling and DoE during process development and data analysis.

6.3 Recommendations for Future Work

6.3.1 pCO_2 Scalability and Robustness

For future work a number of additional studies are required to determine the scalability and robustness of pCO_2 control. The scale-up of pCO_2 control would be important to confirm if pCO_2 control leads to a more scalable and comparable process. However, as mentioned previously this would require a substantial time and cost commitment.

The analysis of the robustness of pCO_2 control is required. This is to determine if pCO_2 control improves batch-to-batch and STR-to-STR comparability. For this analysis, a triplicate run of batches with triplicate runs of pCO_2 conditions would be required, for example, examining three baseline runs with three 140 mmHg conditions in three batches, to determine the inter and intra batch variability. To complete this work the clone stability needs to be understood and controlled, such as using the same age (passage number cells) to minimise the effect.

6.3.2 Carbon Dioxide Design Space

Due to limitations of the pCO_2 online sensors and at-line pCO_2 analytics the maximum pCO_2 level investigated in this study was 140 mmHg. Ideally, a design space for pCO_2 would be generated and values exceeding 140 mmHg would be investigated. However, to do this improved accuracy and robustness of online and at-line pCO_2 analytics is required.

6.3.3 Carbon Dioxide Effect on Product Quality

What has not been determined in the current study is the cause of the effect of pCO_2 and osmolality on the productivity and product quality. For this, analysis of the pH_i and analysis of the internal cell metabolism are required. Analysis of the PTMs that give rise to the basic and acidic variants would also be useful in determining the effect of pCO_2 on the product quality.

6.3.4 Steady State pCO_2 Analysis

Examination under pseudo-steady state would be ideal, such as in a perfusion process, where the effect of interacting parameters can be reduced. Testing conditions under perfusion would also remove the effect of batch-to-batch variability. If a more constant performance (i.e. steady state across time) can be proved in a perfusion system then different conditions can be tested, without the effect of different cell cycles (i.e. growth and stationary phase) interacting. For perfusion processes the effect of pCO_2 control level needs to be examined to determine if the results obtained

in the batch studies apply, as the selection of a constant pCO_2 level is required for a perfusion process.

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8 Appendices

8.1 Flex Quality Control Standards

Parameter	Unit	QC 1 Range	QC 2 Range	QC 3 Range	QC 8 Range	QC 9 Range
pH	-	$6.631 - 6.731$	$6.889 - 6.989$	$7.312 - 7.412$	-	-
pO_2	mmHg	$57.5 - 69.9$	$100.6 - 120.6$	$134.8 - 163.4$	-	-
pCO_2	mmHg	$60.7 - 74.3$	$41.2 - 50.0$	$16.2 - 22.6$	-	-
Glucose	$g.L^{-1}$	$0.75 - 1.25$	$4.25 - 5.75$	$12.75 - 17.25$	-	-
Lactate	$g.L^{-1}$	$0.78 - 1.28$	$2.55 - 3.45$	$4.25 - 5.75$	-	-
NH_4^+	mM	$0.61 - 1.11$	$3.85 - 5.65$	$8.24 - 11.24$	-	-
Na^+	mM	$69.7 - 85.3$	$122.5 - 140.9$	$166.7 - 203.3$	-	-
K^+	mM	$1.71 - 2.29$	$2.58 - 4.42$	$5.55 - 6.85$	-	-
Ca^{++}	mM	$0.50 - 0.74$	$0.67 - 0.93$	$2.25 - 2.85$	-	-
Osmolality	mOsmo.Kg	$217 - 227$	$394 - 406$	$600 - 616$	-	-
Cell Density	$Particles.mL^{-1}$	-	-	-	$5.5 - 6.50$ E5	$5.5 - 6.50$ E6

Figure 8.1 - Quality Control Standards for Nova Bioprofile Flex

8.2 Appendix – Clone Stability Data

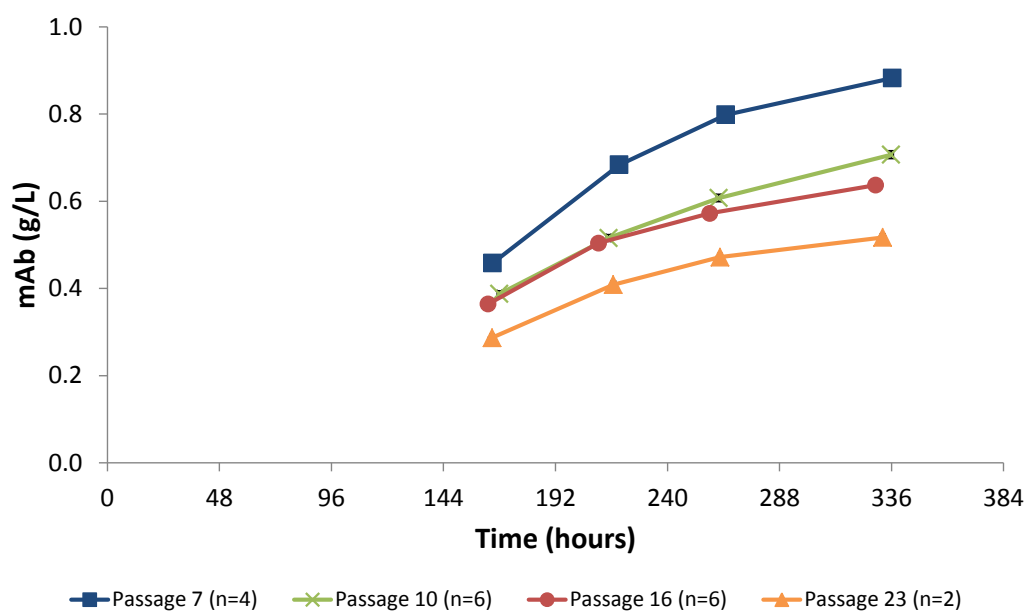Passage 7 (n=4) Passage 10 (n=6) Passage 16 (n=6) Passage 23 (n=2)

Figure 8.2 - Average mAb Titre for Shake Flask Runs in FortiCHO Carried out Under Different Passage Numbers (i.e. culture age) Showing Clone Line Instability (Data courtesy of Alex McLoughlin, Pall Corporation, Shown with S.E. of the mean)

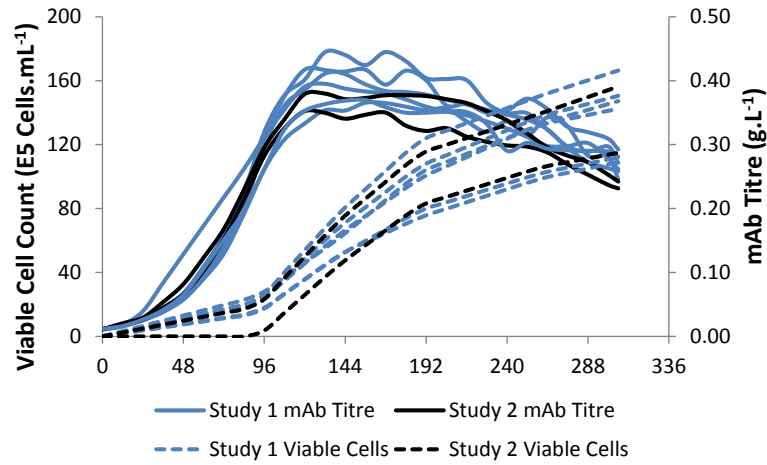## 8.3 Appendix – Baseline Results Comparison

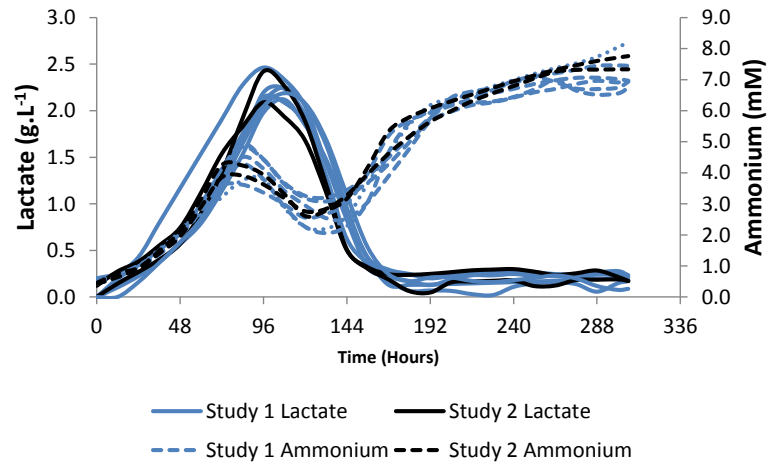**Figure 8.3 - mAb Titre and Viable Cell Titre for Baseline runs in Study 1(Chapter 2) and Study 2 (Chapter 3)**

Figure 8.4 - Lactate and Ammonium Concentrations for Baseline runs in Study 1(Chapter 2) and Study 2 (Chapter 3)

8.4 MPCA Model Data Split Exponential and Stationary

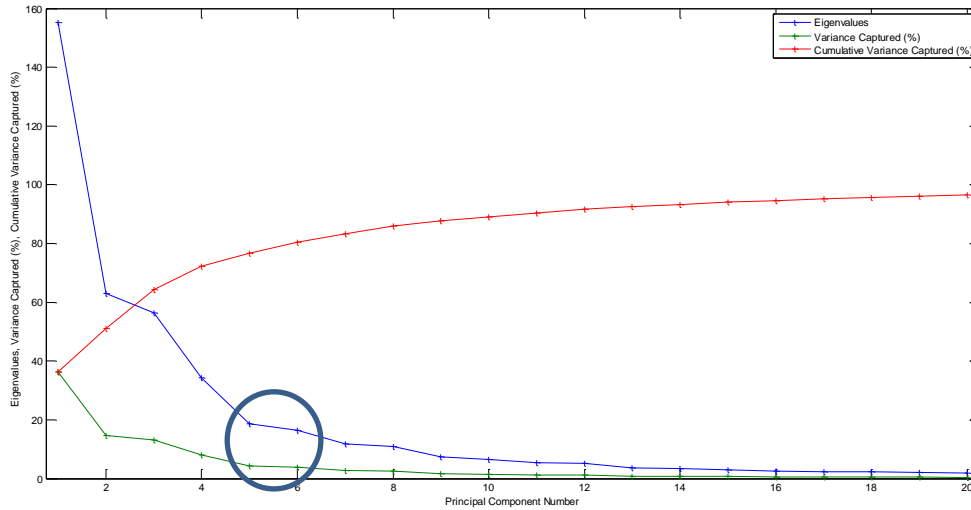### 8.4.1 Selection of Number of PCs for MPCA Model - Nomikos and MacGregor Method

Figure 8.5 - Eigenvalues and Variance Captured versus Principal Component Number for Selection of Number of PCs for Nomikos and MacGregor Method

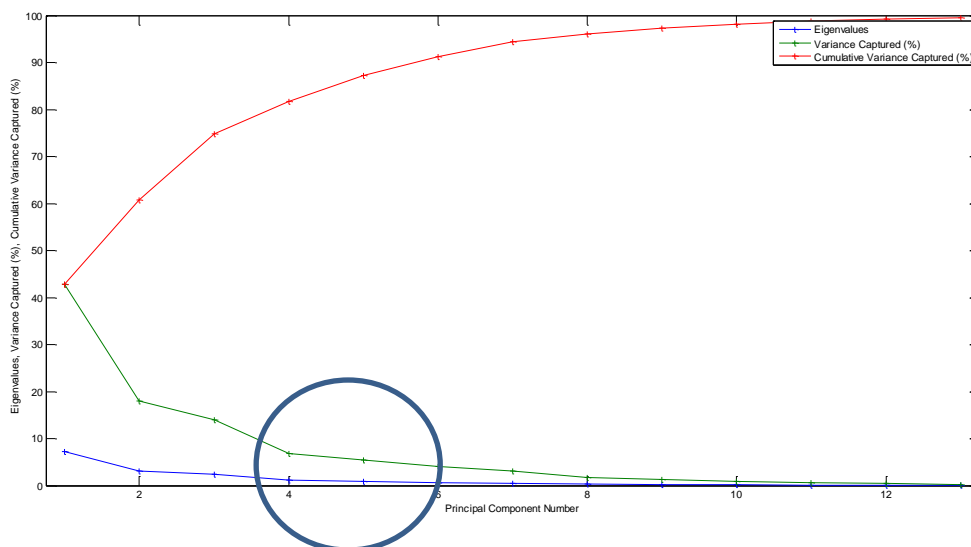### 8.4.2 Selection of Number of PCs for MPCA Method – Wold Method

Figure 8.6 - Eigenvalues and Variance Captured versus Principal Component Number for Selection of Number of PCs for Wold Method

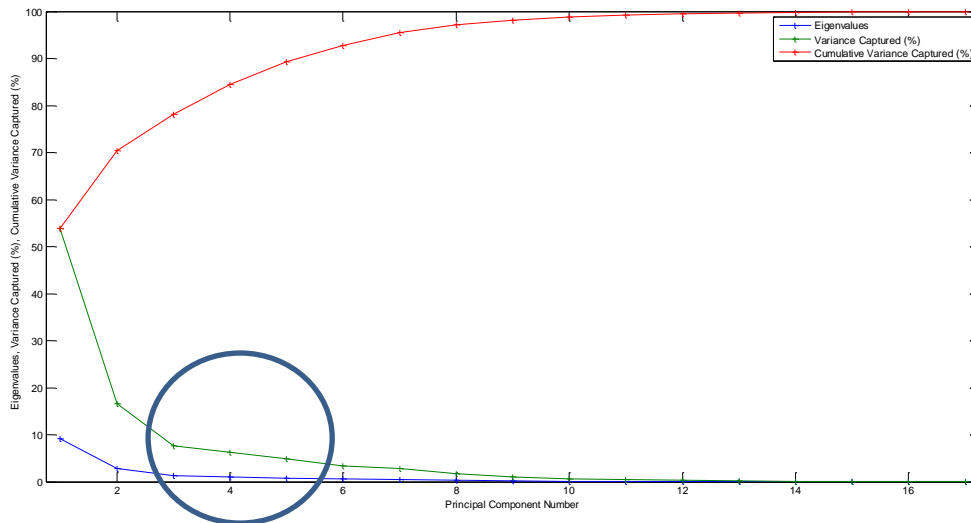### 8.4.3 Selection of Number of PCs for Exponential Phase Model

Figure 8.7 - Eigenvalues and Variance Captured versus Principal Component Number for Selection of Number of PCs for Exponential Phase MPCA Model
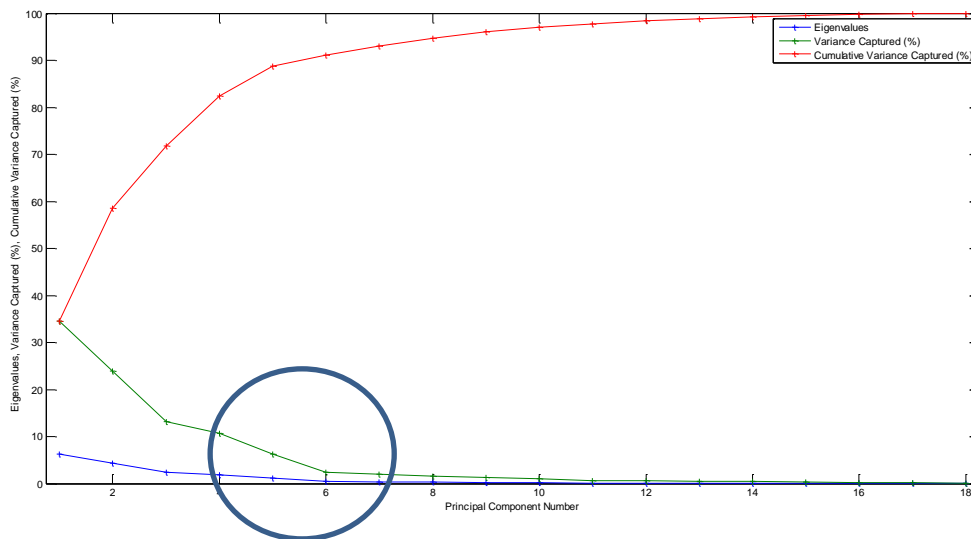
8.4.4 Exponential Phase Model

Figure 8.8 - Eigenvalues and Variance Captured versus Principal Component Number for Selection of Number of PCs Stationary Phase MPCA Model