Abstract

Title of Document: The Effect of Hyper-Osmotic Conditions on the Growth, Metabolism, and Specific Antibody Productivity of a GS-NS0 Cell Line

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The effect of cellular growth, metabolism, and monoclonal antibody production on an industrial GS-NS0 cell line to hyper-osmotic medium was studied. The GS-NS0 cell line was found to have an optimum growth rate at a medium osmolality of 350 mosm/kg and an optimum specific productivity at 450 mosm/kg. Medium osmolality was shown to affect cell size as the cell line exhibited a regulatory cell volume increase response after an initial introduction into hyper-osmotic conditions.

The response of the cell line to an osmotic shift was also studied. Osmolality of the culture medium was increased, at two different time points, through the addition of NaCl. The shift in osmotic pressure was found to have a positive impact on specific productivity of the monoclonal antibody produced.

A finger print of the metabolic response of the GS-NS0 cell line to increased medium osmolality was determined. The application of metabolomics to mammalian cell cultures has not been widely explored. In this study, the cells were quenched and extracted using methods previously developed for microbial and plant cultures. An increase in concentration of internal amino acids, known to be osmolytes, was found under hyper-osmotic conditions.

The Effect of Hyper-Osmotic Conditions on the Growth, Metabolism, and Specific Antibody Productivity of a GS-NS0 Cell Line

By

Stefanie Brady

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Professor Nam Sun Wang, Chair
Professor Ganesh Sriram
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Dedication

I dedicate this thesis to Gerard, the boy who always met me at the bus stop to carry my books.

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Chapter 1 Introduction and Overview

1.1 Problem Description and Motivation

This work focuses on the response of an industrial, monoclonal antibody producing GS-NS0 cell line to increased medium osmolality. In the biopharmaceutical industry, much research is dedicated to learn how the cell's environment, such as osmolarity, temperature, and pH, impact cell growth and antibody production. A clear understanding of the ideal environmental parameters will allow for the optimum production of marketable antibodies. The osmolarity of a cell's environment greatly impacts the metabolism of the cell and in turn the cell's ability to produce antibodies. Osmolarity can be easily and cheaply altered during industrial production, thus knowledge of how osmolarity can positively impact antibody production can be financially beneficial for a company.

Osmolarity (or osmolality) is a measurement of the moles of a solute that contribute to the osmotic pressure of a solution, thus if a solution consists of 1 mol/L of glucose, the osmolarity would be 1 osmol/L. However, in the case of ionic compounds which disassociate in water, the osmolarity is determined by the number of disassociated species; therefore, 1 mol/L of NaCl would correspond to an osmolarity of 2 osmol/L. Osmolarity is measured in the units of osmoles per unit volume, while osmolality is measured in the units of osmoles per unit mass. The analytical equipment used during the execution of this work calculated the solute concentration in terms of

osmolality (osmoles per unit mass), thus osmolality will be used throughout the rest of this thesis.

1.2 Significance

This thesis work can essentially be divided into three topics each with their own significance. The first set of experiments (Chapters 4 and 5) give an insight into the industrial cell lines response to hyperosmotic conditions. At HGS, research into a cell line's response to hyperosmolarity had not been previously examined. While the results are similar to findings reported for other cell lines, this experimentation gave us the ability to gain metabolic parameter data pertinent to a developmental cell line.

The second set of experiments (Chapter 6) looked at altering the cellular environment in order to increase antibody production. It was found that increasing the osmolality of the cell culture during the production process can be beneficial to the antibody production rate.

The last experiment (Chapter 7) examined the use of metabolomics to further understand the effect of osmolality on the intracellular metabolites of the cell. This technique is not routinely performed on mammalian cell culture. In these experiments, we found that the intracellular metabolite concentration was indeed impacted by the environmental osmolality. The findings correlate to osmotic responses seen in other cell types.

1.3 Overview of Chapters

Chapter 2 covers a brief background divided into three sections: 1. monoclonal antibodies, 2. osmolality effects on cell culture, and 3. metabolomics. In the monoclonal antibodies subsection, information on the structure and function of antibodies is described. An overview of the industrial cell lines used to produce antibodies is given as well as marketing economics of antibodies as therapeutics. In the second subsection, a brief overview of the general osmolality effects on cell culture is introduced. Further discussion of the impact of osmolality on metabolism and antibody production is given in the introduction section of the subsequent chapters describing the experimental research. In the third background section, a brief introduction to metabolomics and the tools used for this analysis is described.

Chapter 3 describes the cell line, medium, and analytical methods used throughout the cell culture experiments.

Chapters 4 through 7 cover the different experiments performed. Each chapter includes an introduction with background information relevant to the topic as well as the methods used to perform the experiment, and the results, discussion, and conclusions for each experiment.

Chapter 8 concludes the highlights and overall findings of this research.

The last chapter, 9, discusses future studies that can be performed to gain additional insight into this topic.

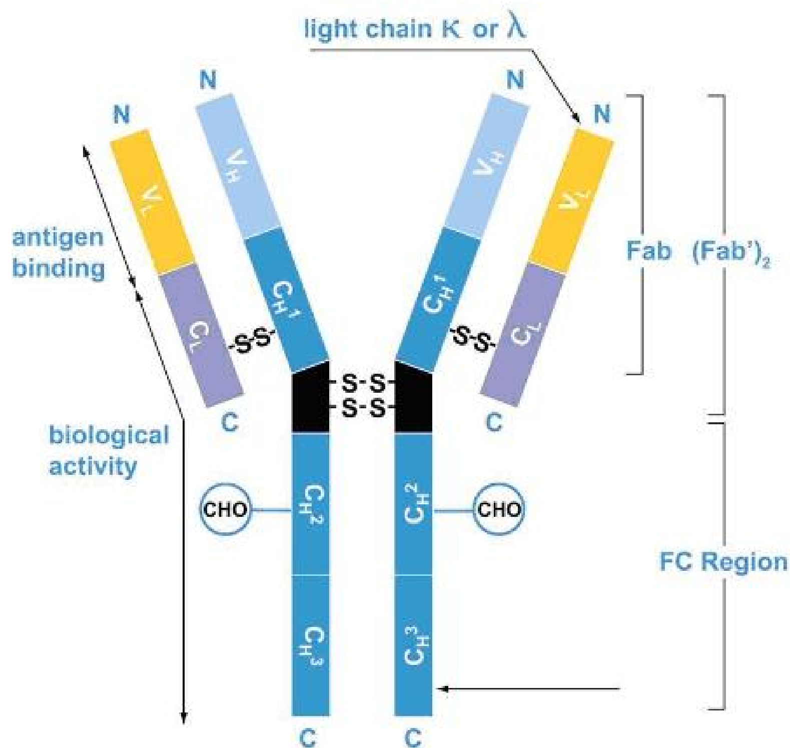
Chapter 2 Background

2.1 Monoclonal Antibodies

2.1.1 Antibody Structure and Function

Antibodies are produced by the body as a response to an antigen, a foreign particle, or infectious agent. After the introduction of an antigen, B lymphocyte cells multiply and produce an antibody to the antigen. The antibody contains a specific binding site that recognizes a specific site on an antigen, termed an epitope. Monoclonal antibodies (mAbs) are antibodies that are produced from one particular B lymphocyte and its cloned cells. Monoclonal antibodies are specific to one epitope region on the antigen. However in an immune response in the animal body, multiple different B lymphocyte cells produce different antibodies, which bind to different regions on the antigen. The collection of all the antibodies produced due to the immune response is termed polyclonal antibodies (Lodish, 2000).

Antibodies consist of two large (heavy, H) and two small (light, L) polypeptide chains which are paired together as shown in Figure 2-1. Sequence analysis of antibodies found that both the heavy and light chain comprise of regions of variable sequence at the N-terminus and constant regions at the C-terminus (Karu, 1995). Antibodies have been divided into subclasses based on the structure and function of the constant regions.



Taken From: http://www.abcam.com/index.html?pageconfig=resource&rid=11258&pid=11287

Figure 2-1. Antibody structure

Digestion of the antibody by the proteolytic enzyme papain found that three fragments are produced. Two of the fragments retain the antigen binding region and are termed Fab fragments (Elgert, 1996). The third fragment, termed Fc, consists of the heavy chain constant regions, CH2 and CH3 (Figure 2-1).

Further digestion of the Fab fragments can generate Fv fragments which consist of only the variable domains of the heavy and light chains (Figure 2-2). Fv fragments are the smallest antibody structure than can preserve the antigen binding region (Karu, 1995). It is these variable regions which give the ability for immune responses to generate antibodies to different antigens (Kim, 2005).

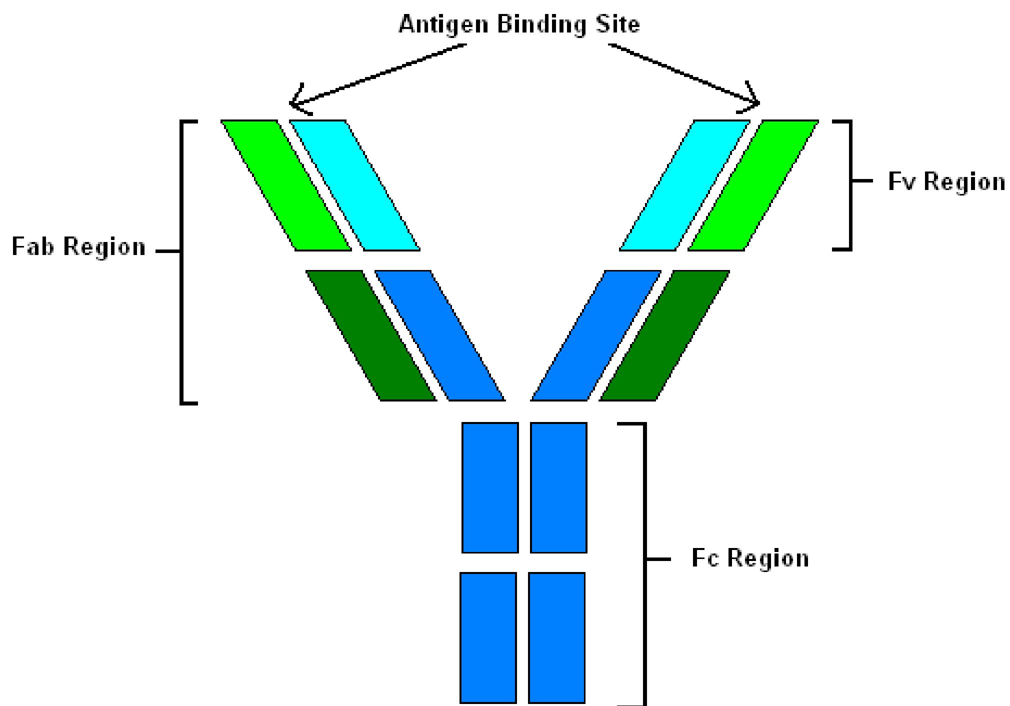


Figure 2-2. Antibody fragments

The variable region of both the heavy and light chains is further divided into regions called hypervariable or complementarity-determining regions (CDR). The CDR regions are linked together by intervening sequences termed the framework residues (FR) (Figure 2-3). Framework residues compose of approximately 85% of the variable region (Elgert, 1996). When the light chain and heavy chain bind together, the CDR regions form a cleft, which forms the antigen binding site (Figure 2-4).

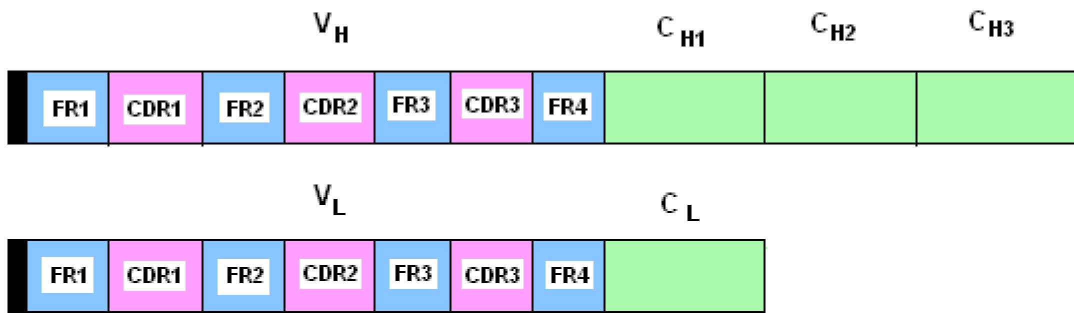


Figure 2-3. Location of the FR and CDR regions on the Fab

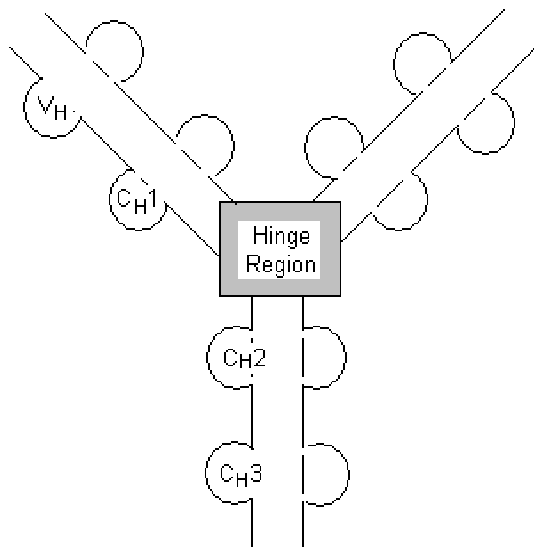


Figure 2-4. CDR regions of antibodies

2.1.2 Antibody Engineering

In 1975, Kohler and Milstein described a method for making cell lines for secreting monoclonal antibodies (Kohler, 1975). Normal antibody producing mouse spleen cells are fused to myeloma cells in order to produce an immortal clone termed a hybridoma (Lodish, 2000). However, the mouse antibodies when introduced to humans caused immunogenic issues which limited their use as a therapeutic (Spier, 2000). In 1984, Morrison *et al.* described the genetic engineering of chimeric mAbs,

a fusion of a mouse variable region with a human constant region. These mAbs have also seen an immunogenic response in humans (Kim, 2005).

Due to immunogenic responses, the use of more human-like mAb fragments was desired for therapeutics. CDR-grafting is used to produce “humanized” mAbs, where the CDR loops that are responsible for antigen binding are grafted onto human FR. However, CDR-grafting has often resulted in loss of the affinity for the humanized mAb to bind to the antigen (Kim, 2005).

The next move for antibody engineering was the development of a fully human mAb. Today, fully-humanized mAbs are mostly generated by one of two procedures: 1. phage display technology (Hudson, 2003), or 2. XenoMouse technology (Jakobovits, 2007).

2.1.3 Cell Line Selection and Formation

To express the developed antibody, the DNA needs to be added to a plasmid and in turn introduced into a cell line. The most common cell lines used for antibody expression in industry are from yeast, mouse, or human sources. However, mammalian cell lines are used more frequently to express antibodies as therapeutics, due to glycosylation patterns formed during post-modification of proteins. The mammalian cell lines commonly used in industry include Chinese hamster ovary cell line, CHO; non-secreting mouse myeloma cell line, NS0; and a human retina derived cell line, Per.C6 (Table 2-1).

Table 2-1. Pros and cons of cell line source

Characteristic	CHO	NS0	Per.C6
FDA Approved mAbs	Yes	Yes	None
Glycosylation	Human-like	Human-like	Human
Human Viral Issues	None	None	Yes
Cholesterol Dependence during Cell Culture Process	None	Yes	None

2.1.4 Market for Industrial Produced Monoclonal Antibodies

The industrial production of mAbs is a multi-billion dollar worldwide market. Between 2001 and 2002, the global therapeutic mAb market was $5.4 billion (Reichert, 2004). In 2006, the market grew to over $20 billion (Maggon, 2007). The mAbs tend to be developed for chronic conditions such as cancer and autoimmune disorders as well as infectious diseases (Table 2-2). These indications and the low potency of mAbs result in large doses and high costs (upwards of $35,000) for the patients (Farid, 2007).

To reduce the treatment cost for the patient, industry needs to reduce manufacturing costs from its current $1000 per gram to a more economical $100 per gram or less (Farid, 2007). In order to decrease manufacturing costs, companies try to produce mAbs at the largest scale possible. Companies such as Genentech and Amgen are running commercial bioreactors at the 20,000 L scale. However, increasing the bioreactor size does not necessarily reduce patient cost significantly. The cost of goods for manufactured mAbs is still reported to be 20-25% of sales (Farid, 2007). Developing ways to reduce the cost of goods could help to drastically reduce the patient cost drastically.

Table 2-2. Therapeutic mAbs approved in the United States*

Company	Generic Name	US Trade Name	mAb Type	Indication
Johnson & Johnson	Muromonab-CD3	Orthoclone OKT3	Murine	Transplantation
Centocor	Abciximab	ReoPro	Chimeric	Cardiac ischemic
Biogen IDEC	Rituximab	Rituxan	Chimeric	RA, NHL
Protein Design Labs	Daclizumab	Zenapax	Humanized	Prophylaxis
Novartis	Basiliximab	Simulect	Chimeric	Prophylaxis
MedImmune	Palivizumab	Synagis	Humanized	RSV
Centocor	Infliximab	Remicade	Chimeric	RA
Genentech	Trastuzumab	Herceptin	Humanized	Caner
Wyeth	Gemtuzumab	Mylotarg	Humanized	Leukemia
Millinnium/ILEX	Alemtuzumab	Campath	Humanized	Leukemia
Biogen IDEC	Ibritumomab tiuxetan	Zevalin	Murine	NHL
Abbott	Adalimumab	Humira	Human	RA
Genentech	Omalizumab	Xolair	Humanized	Asthma
Corixa	Tositumomab-I131	BEXXAR	Murine	NHL
Genentech	Efalizumab	Raptiva	Humanized	Psoriasis
Imclone Systems	Cetuximab	Erbitux	Chimeric	Skin Cancer
Genetech	Bevacizumab	Avastin	Humanized	Cancer

* Based on (Reichert, 2004)

A majority of commercial cell lines produce mAbs at the 1 – 2 g/L concentration. If a cell line's productivity could be doubled, the cost of goods for the commercial production could be reduced in half. Process development efforts, such as, determining process operation set points, batch and feed medium composition, and feeding strategies, can drastically increase productivity. Typical mammalian culture utilizes process operation set points that mimic the intracellular conditions of the mammalian cell: pH of 7, temperature of 37°C, and osmolality of 290 – 320 mosm/kg. Deviations from the intracellular conditions have been shown to produce stresses on the mammalian cell culture and these stresses have been shown to increase mAb yields (Hendrick, 2001; Osman, 2001).

2.2 Osmolality Effect on Cell Culture

Typically, cells have an internal osmolality of 270 – 300 mosm/kg (Duncan, 1997). Mammalian cells maintain their internal osmolality through osmoregulation. If a cell is introduced into a hypo-osmotic environment, water will move into the cell; if a cell is placed in a hyper-osmotic environment, water will move out of the cell into the solution with a greater ionic strength (Alberts, 1994). In both cases, the cell quickly acts to return the internal osmolality to natural conditions by controlling the uptake and release of ions and charged molecules.

Cells contain the ability to regulate their cellular volume. Changes in cell volume (size) are mainly caused by osmotic pressure gradients across the cell membrane (Lang, 1998 a.). When cells are exposed to hypo-osmotic conditions, they will initially swell and when exposed to hyper-osmotic conditions, they will initially shrink. In both cases, the cells will eventually return to the original cell volume through regulatory cell volume decrease (RVD) or increase (RVI) (Lang, 1998 b.; Erickson, 2001). Since the increase or decrease of ions, Na^+, Cl^-, $HCO3^-$, K^+, contributes to the osmotic pressure gradients across a membrane, ion transport across the cell membrane is of great importance in regulation of cell volume (Lang 1998 a.).

The accumulation of electrolytes, during RVI, after cell shrinkage, is limited because high ion concentrations interfere with the structure and function of proteins in the cell (Yancey, 1982). It has been reported that levels of intracellular K^+ vary slightly despite large changes in medium osmolality (Lin, 1999 b.). For a cell to return to its original volume, cells produce or uptake osmolytes, molecules which create osmolality without compromising cell function (Handler, 1993; Kinne, 1993), as a

secondary means of osmotic control. Osmolytes shown to be key to regulating cell volume include, polyalcohols, such as sorbitol and inositol, methylamines, such as betaine, and amino acids and amino acid derivatives, such as glycine, glutamine, glutamate, aspartate, and proline (Burg, 1994; Garcia-Perez, 1991; Kinne, 1993; Lang 1998 b.; Chua, 1994). The cellular accumulation of osmolytes for cells cultured in hyper-osmotic conditions has been shown to help them proliferate in the environment (Yancey, 1990).

2.3 Metabolomics

Process development improvements to increase cell growth and productivity have mainly been from hit or miss experimentation or design of experiment analysis by altering growth conditions. Advances in systems biology techniques, such as metabolomics, the quantification of intracellular metabolites, can increase the understanding of how these process changes enhance cellular performance. Metabolomics can provide a deeper insight into relevant biochemical pathways and their regulations; this can lead to improved production processes by increasing mAb formation and reducing byproduct accumulation. Metabolomics can provide a clearer understanding of substrate consumption, product formation, and cellular energy management which can increase productivity and subsequently reduce mAb production costs (Oldiges, 2007). Overall the application of metabolomics in the area of mammalian cell culture is relatively undeveloped (Khoo, 2007).

The first step of metabolomics, quenching of the cell's metabolism, is the most crucial to ensuring an accurate profile of the intracellular metabolites. Quenching is the rapid stopping of the enzymatic activity and subsequent changes to the metabolic levels.

Currently, there is no established method for quenching mammalian cells in suspension culture (Oldiges, 2007). Basic techniques for quenching metabolism in other cell types include chilling the sample in cold methanol (Villas-Boas, 2005), snap-freezing in liquid nitrogen (Griffin, 2000) and acid treatments (Mashego, 2007). These methods tend to be time consuming and laborious and can lead to a degradation of the metabolites. Methods such as spray freezing into cold methanol during continuous sampling (Buchlolz, 2002) can expedite the sampling and quenching process. Recently, Wiendahl et. al (2007) has designed a micro heat exchanger to quench the metabolism of mammalian cells in order to improve the sampling procedure and obtain better quantification of metabolites.

After quenching, the next step is extracting the intercellular metabolites. Frozen cell samples are either homogenized or sonicated in the presence of organic and/or non-organic solvents in order to release the polar and non-polar metabolic compounds (Khoo, 2007). The mixture of cell debris and extracted metabolites is then centrifuged or filtered to separate the liquid containing the desired metabolites.

Once the metabolites have been extracted from the cells they are next separated and identified. Traditional procedures of gas-chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometery (LC-MS) and new techniques of capillary-electrophoresis mass spectrometry (CE-MS) and Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) are being used to rapidly identify and quantify the 100s and 1000s of metabolites present in a sample (Fernie, 2004).

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 GS-NS0 Cell Line

A cholesterol dependent, GS-NS0 mouse myeloma cell line producing a human mAb was used for the execution of these experiments. The GS-NS0 cell line was transfected with the glutamine synthetase selectable marker. Glutamine synthetase is an enzyme that catalyzes the conversion of glutamate and ammonia to glutamine. NS0 cell lines containing this selectable marker are grown without the presence of glutamine.

3.1.2 Growth and Production Media

A commercially available basal growth medium without L-glutamine was used as the batch medium. The commercial medium is protein-free and chemically defined and contains a proprietary blend of glucose, amino acids (except L-glutamine), and salts that have been shown to support growth in hybridoma and myeloma cell lines. Since the GS-NS0 cell line used for these studies was cholesterol dependent, a concentrated cholesterol suspension was added to support cell growth.

For fed-batch cultures, a proprietary complex feed medium (HGS) was used to support cell growth beyond the batch phase. The feed medium was supplemented with cholesterol which is required for the cell line.

3.2 Analytical Methods

3.2.1 Cell Density and Cell Viability

Cell density and cell viability were measured using a CEDEX Cell Counter (Innovatis). The CEDEX cell counter performs an automated trypan blue exclusion assay on a cell culture sample. The instrument takes multiple images of the cell culture sample and then quantifies the live (non-stained) and dead (stained) cells. A total cell number and cell viability is determined for each image by the ratio of live cells to total cells. A cell culture sample's viable cell density (VCD), total cell density (TCD), and viability is reported as an average of the values of all the images taken.

3.2.2 Glucose and Lactate Measurements

Glucose and lactate concentrations in the culture medium were measured using an YSI 2700 (Yellow Springs International). The YSI 2700 uses an enzymatic reaction to determine the concentration of glucose or lactate in the sample. An immobilized enzyme, either glucose oxidase or lactate oxidase, is located between two membranes. Glucose (or lactate) is then oxidized after it crosses the membrane and produces hydrogen peroxide. The hydrogen peroxide is subsequently oxidized and produces a current that is proportional to the concentration of the glucose or lactate concentration in the cell culture sample.

3.2.3 Osmolality

Osmolality was measured using an Advanced 2020 Multi-sampler Osmometer (Advanced Instruments). This osmometer measures the osmolality (concentration of

particles per mass of solution) by the freezing point depression method. The extent to which a solvent's freezing point has been decreased is a direct measure of the concentration of particles in the solution.

3.2.4 pH

pH measurements were conducted with a Rapid Lab 240 Blood Gas Analyzer (BGA) (Bayer). The BGA is primarily used in the healthcare industry to measure the concentration of pO_2 and pCO_2 as well as pH in blood samples; however it has routine use in the biopharmaceutical industry to measure these quantities in mammalian cell culture samples.

3.2.5 mAb Concentration

The concentration of mAb in cell culture samples was determined by a high performance-liquid chromatography (HPLC) method using an affinity column. The mAb binds to an antigen that is attached to the column beads. The antibody is then eluted off the column and the concentration of the mAb peak is determined by the absorbance at the 280 nm wavelength.

Chapter 4 Effect of Osmolality on Cell Growth, Glucose Consumption, and Lactate Production

4.1 Introduction

Typical cell culture medium has an osmolality of 270 to 300 mosm/kg (Duncan, 1997). Under these conditions, the cell culture tends to have the highest growth rate, μ. Hyper-osmotic conditions have been shown to decrease μ (Chua, 1994; Wu, 2004; Ozturk, 1991) and increase glucose consumption rates, ν_G and lactate production rates, q_L (Table 4-1).

To improve our understanding of the effect of medium osmolality on cell growth and metabolism of a GS-NS0 cell line at HGS, we evaluated μ, ν_G, and q_L for cultures grown at five different osmolalities.

Table 4-1. Effect of osmolality on metabolic parameters

Cell Line	Osmolality, mosm/kg	Glucose Consumption Rate, ν_G	Lactate Production Rate, q_L	Reference
Mouse Hybridoma (AFP-27)	300 357 400	0.245 0.255 0.300	0.225 0.240 0.260	Lin, 1999 (b)[1]
Mouse Hybridoma (167.4G5.3)	290 362 435	0.247 0.342 0.579	0.396 0.553 0.949	Ozturk, 1994
Mouse Hybridoma (6H11)	330 450	0.294 0.267	0.644 0.622	Oyaas, 1994

[1] Values shown in Table 4-1 are interpolated from figures from Lin, 1999 b.

In this experiment, the effect of medium osmolality on the growth rate, glucose consumption, and lactate production rates of the GS-NS0 cell line was determined. Medium osmolalities of 290, 320, 350, 400, 450 mosm/kg were tested. The osmolality of the commercial basal medium manufactured was 180 mosm/kg. NaCl was supplemented to the medium to achieve the desired osmolality. Previous studies have shown that the effect of medium osmolality on cell growth and metabolism was independent of electrolyte used to increase osmolality (Chua, 1994).

4.2 Methods

Basal medium was prepared with five different osmolalities, 290, 320, 350, 400, and 450 mosm/kg, by adjusting the concentration of NaCl in the medium. Synthetic cholesterol was added to the basal medium.

A GS-NS0 cell culture, initially grown at approximately 300 mosm/kg, was obtained for use as an inoculum for this experiment. The inoculum culture was added to each medium at a starting viable cell density (VCD) of 2.0 x 10^5 cells/mL. The cultures were grown in 250 mL shake flasks with a working volume of 125 mL. Operating conditions for the incubator were: 115 rpm, 37°C, 80% relative humidity (rh), and 5% CO_2. Once the fastest growing condition reached approximately 18 to 20 x 10^5 cells/mL, all the shake flasks were passaged into fresh medium of the same osmolality. Four passages were performed. Each condition was performed in duplicate.

Samples were taken daily to measure VCD, viability, glucose and lactate concentration, pH, and osmolality of the cultures. The time course data was used to

calculate the following metabolic parameters during exponential growth: glucose consumption rate, lactate production rate, and cellular growth rate.

4.3 Results

The medium with the highest osmolality (450 mosm/kg) was shown to have the largest impact on the VCD of the cultures (Figure 4-1). The cultures grown at 350 mosm/kg were shown to produce the highest maximum VCD.

The viability of the culture (Figure 4-2) was not affected by osmolality up to 400 mosm/kg, thus the increase in osmolality, for the range tested, does not promote cell death or apoptosis. The viability of the GS-NS0 cells cultured at 450 mosm/kg was reduced; however upon the fourth passage, the viability was similar to other conditions. This indicates that the cells were capable of adapting to the higher osmolality after increased exposure.

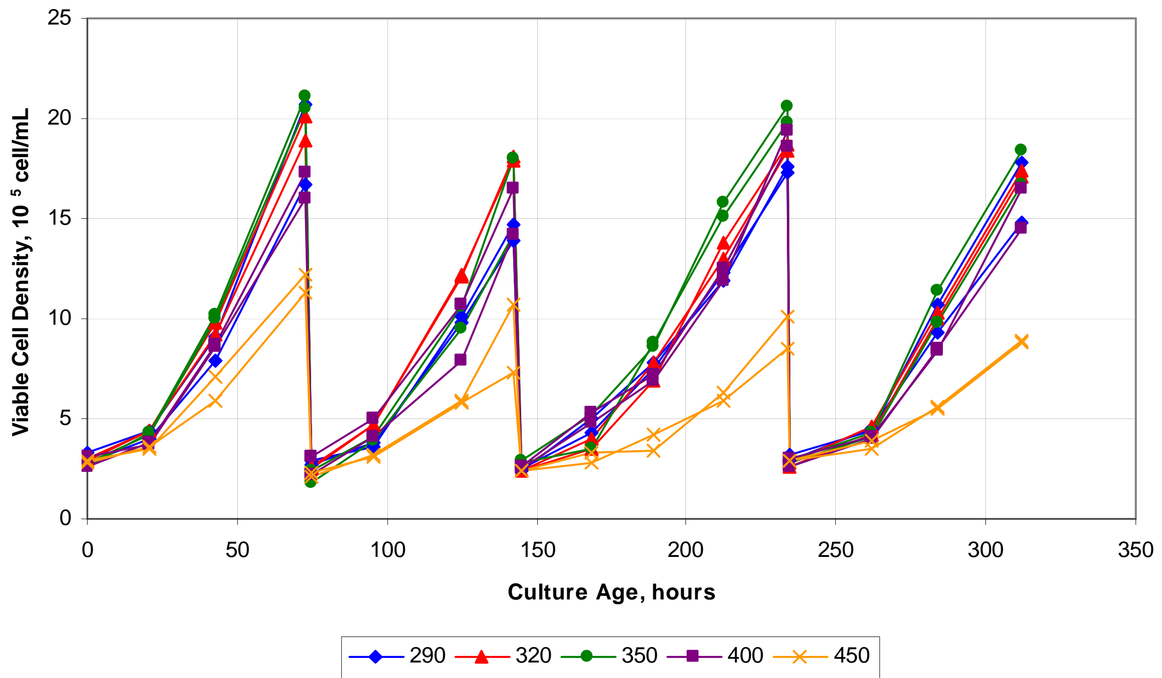


Figure 4-1: Osmolality effect on VCD for subsequent passages

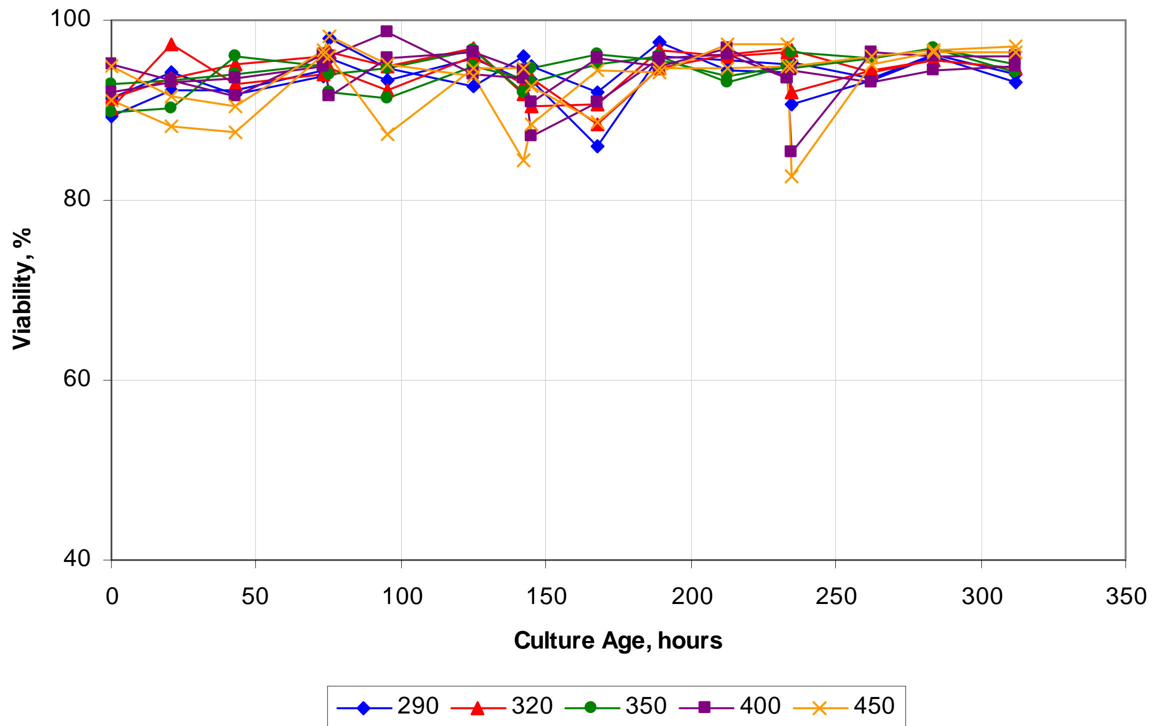


Figure 4-2: Viability of the cultures during subsequent passages

The growth rate of the GS-NS0 cell line was found to be the highest in the 350 mosm/kg medium (Figure 4-3). Overall, the growth rate was significantly less in the 450 mosm/kg medium. Upwards of a 30% difference in growth rate can be seen between passages for the same osmolality condition; however, the effect of medium osmolality on growth rate remains independent of passage number. These differences can be contributed to differences in the inoculation VCD and the inherent variability of cell culture processes.

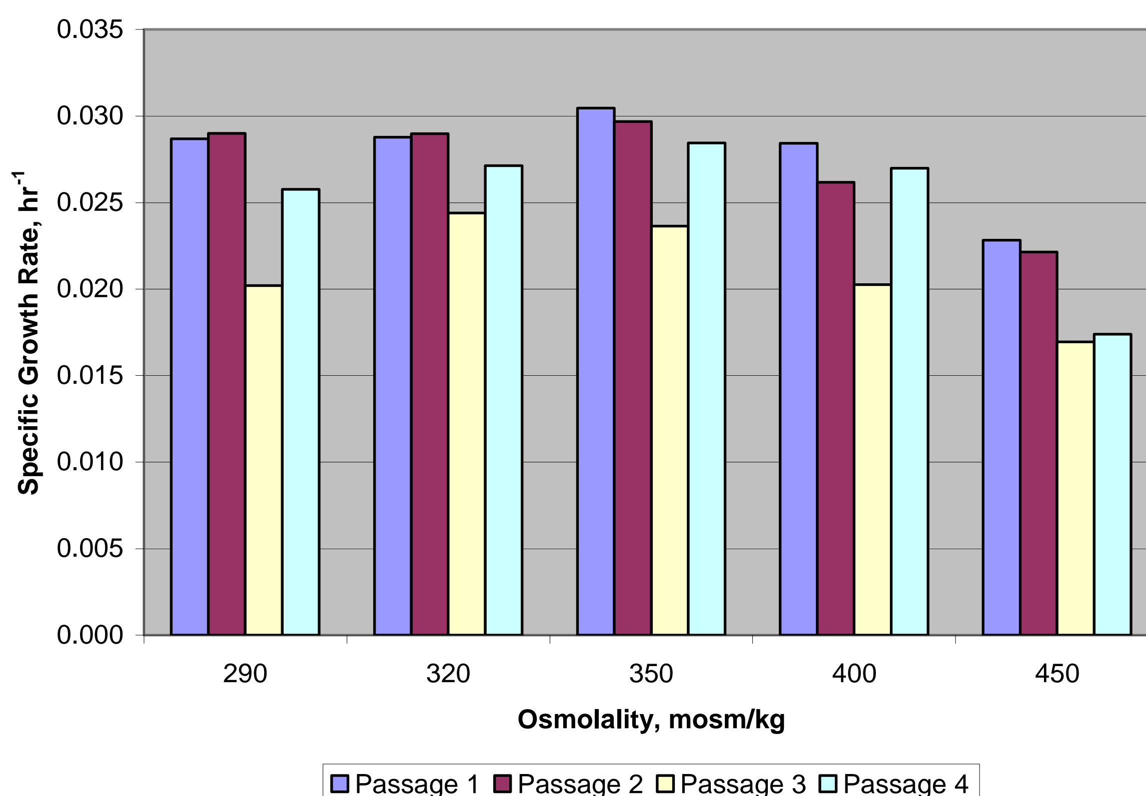


Figure 4-3: Osmolality effect on growth rates during batch phase.

Figure 4-4 shows the glucose and lactate trends for the passages 1, 2, and 3. The cultures grown at hyper-osmotic conditions consumed the greatest concentration of glucose and produced the highest concentration of lactate. As the osmolality of the culture medium increased, so did these metabolic rates (Figure 4-5 and Figure 4-6).

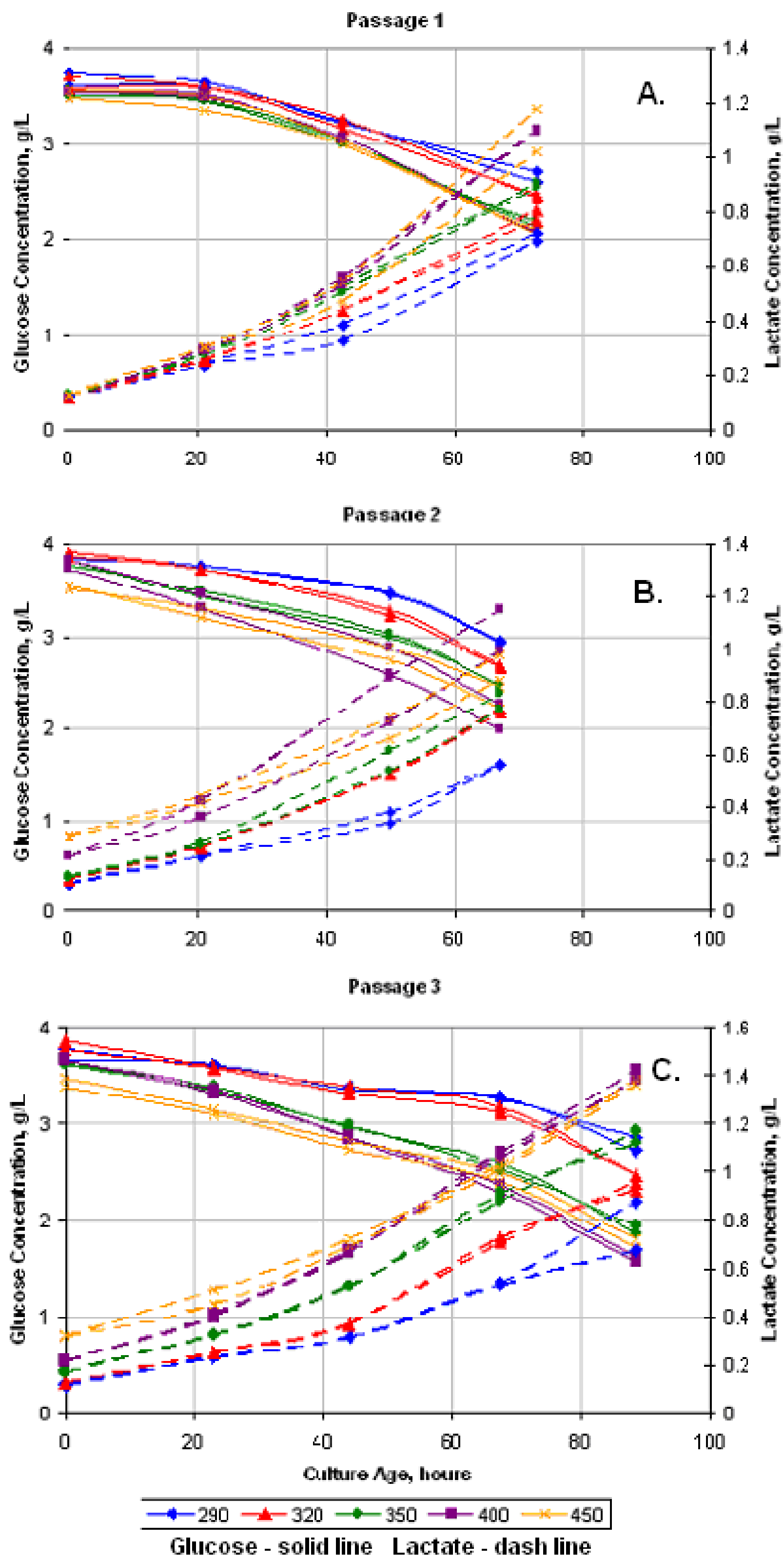


Figure 4-4. Glucose and lactate trends during passages 1 – 3

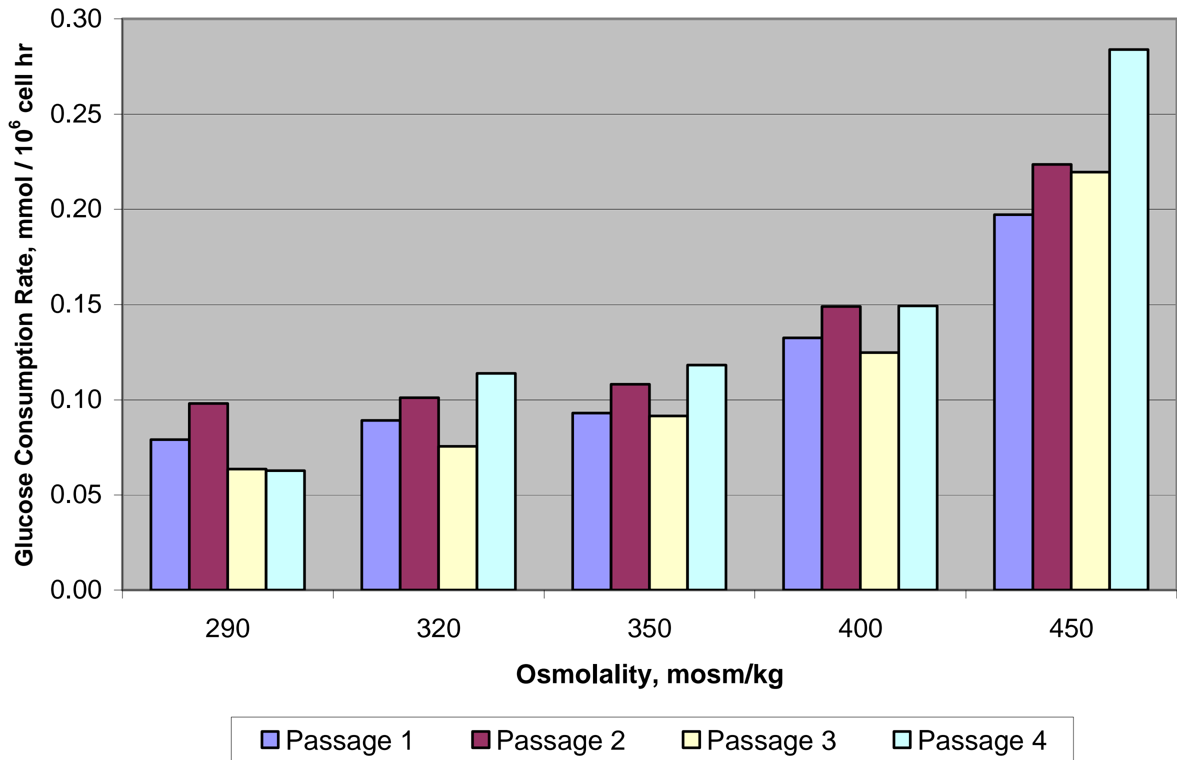


Figure 4-5: Osmolality effect on glucose consumption rates during batch phase.

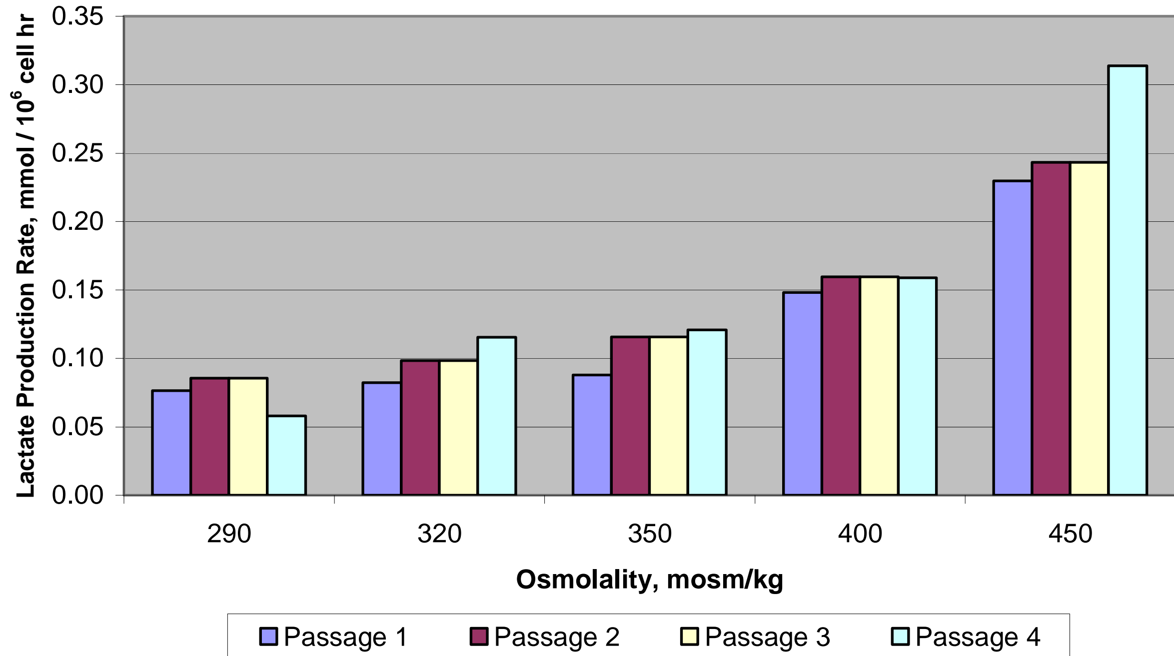


Figure 4-6: Osmolality effect on lactate production rates during batch phase.

The average μ, ν_G, and q_L across the passages, for each osmotic condition was calculated and the yield of lactate from glucose was determined (Table 4-2). The grams of lactate produced per gram of glucose, $Y_{L/G}$, was found to increase throughout with increasing osmolality. This suggests that the cells were stressed at higher osmolalities, as higher amounts of lactate were produced per gram of glucose.

Table 4-2. Specific growth and metabolic rates during batch phase

Metabolic Parameter	290 mosm/kg	320 mosm/kg	350 mosm/kg	400 mosm/kg	450 mosm/kg
μ, hr^{-1}	0.0259	0.0273	0.0286	0.0255	0.0198
ν_G, $\mu mol/10^6$ cell / hr	0.0853	0.0949	0.1027	0.1388	0.2311
q_L, $\mu mol/10^6$ cell / hr	0.0852	0.0987	0.1101	0.1567	0.2575
$Y_{L/G}$, gram lactate produced / gram glucose	0.999	1.040	1.071	1.129	1.114

When the cells were initially introduced into the different osmolality media, the cell diameter was shown to decrease in correlation with increased osmolality (Figure 4-7). The 450 mosm/kg cultures were found to be 9% smaller than the cultures inoculated into iso-osmotic medium. After 24 hours, the cell diameter of all the cultures had increased from the initial decrease. At 48 hours, a clear correlation between cell diameter and medium osmolality was found; as the osmolality of the medium increased, so did the cell's diameter.

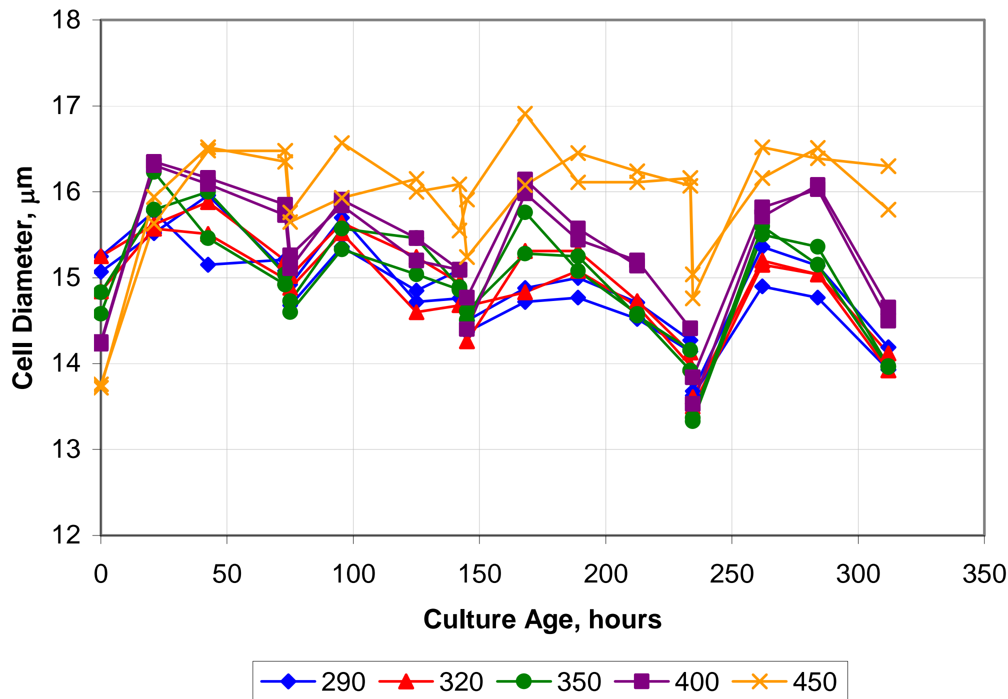


Figure 4-7: Osmolality effect on cell diameter

4.4 Discussion

The osmolality of the cell culture medium greatly affected the growth rate, glucose consumption rate, and lactate production rate of the GS-NS0 cell line (Table 4-2). Cultures grown at 350 mosm/kg were found to have the highest growth rate. This finding is surprising as cells tend to have an internal osmolality of 270 – 300 mosm/kg (Duncan, 1997). The glucose consumption rate and lactate production rates increased as the osmolality of the medium increased. In the hyper-osmotic cultures, the increase in glucose consumption was not routed to increasing cellular mass. Instead, the cells showed a stress response to hyper-osmotic conditions as $Y_{L/G}$ increased with increasing osmolality. Previous research has suggested that cultures grown under hyper-osmotic conditions have an increase in ATP production (Lin, 1999

b). The increase in glucose consumption could be correlated with an increase in energy requirements needed for cells to proliferate in the hyper-osmotic medium. The effect of medium osmolality on growth rate, glucose consumption and lactate production rates correlate with previous data reported by Lin (1999 b.) and Ozturk (1994).

The hyper-osmotic medium did not affect the viability of the GS-NS0 culture (Figure 4-2). This finding is similar to results, presented by Oyaas (1994), using a hybridoma cell line. Oyaas (1994) showed that the death rate, k_d, for cultures grown between 330 to 465 mosm/kg remained unchanged.

The GS-NS0 cell line was found to possess cell volume regulatory mechanisms. When the cells were first introduced to hyper-osmotic conditions, the cells initially shrunk as a response to the osmotic pressure gradient and the loss of water (Figure 4-7). These findings correlate with data presented by Ozturk and Palsson (1991), where the hybridoma cell line they studied showed a similar decrease in cell volume after initial exposure to increased medium osmolality.

However, after the initial decrease in diameter, the cells began to increase in size, as shown by the measurement at 24 hours (Figure 4-7). These findings suggest that the cells regulate their volume based on the regulatory cell volume increase (RVI) mechanism described by Lang (1998 a.). RVI proposes that cells initially shrink when exposed to hyper-osmotic conditions but then approach the original cell volume in attempts to return to a steady state. Lang (1998 a.) reported that in order for a cell to return to a more natural state, they need to uptake electrolytes and osmolytes.

Overall, the cells grown at the highest osmotic conditions became the largest size through the use of regulatory mechanisms. These findings are comparable to previous research on hyper-osmotic stress responses on a hybridoma cell culture (Ozturk, 1991).

Chapter 5 Effect of Osmolality on Monoclonal Antibody Production

5.1 Introduction

Hyper-osmotic growth conditions have shown to produce higher mAb titers, over iso-osmotic conditions, in numerous cell lines (Table 5-1). Some studies have suggested the increase in ATP production in hyper-osmotic medium could be the reason for increased mAb production (Lin, 1999 b.). However, the increase in mAb production does not necessarily correlate to an increase in total mAb produced, since the cell growth rates are reduced in the hyper-osmotic medium (Table 5-1, Figure 4-3). Further knowledge of how osmotic pressure affects the cellular metabolism will provide insight into an economical means of increasing industrial antibody production (Wu, 2004).

To improve our understanding of how medium osmolality effects the mAb production of a GS-NS0 cell line at HGS, we evaluated mAb production under five different osmolalities.

Table 5-1. Osmolality effect on mAb production and growth rate from published

Cell Line	Osmolality, mosm/kg	Change in mAb Production, %	Change in Growth Rate, %	Reference
Mouse Hybridoma (167.4G5.3)	398	+ 59.1	- 30.0	Ozturk, 1991
	435	+ 104.5	- 40.0	
Mouse Hybridoma (2HG11)	400	+ 98.7	- 33.3	Chua, 1994
	450	+ 97.4	- 58.3	
GS-NS0 (6AI(100)-3)	425	+ 376.0	- 20.0	Wu, 2004

5.2 Methods

Prior to the inoculation of shake flasks for the fed-batch experiment the cells were acclimated to different osmolalities as a result of three passages at 290, 320, 350, 400, and 450 mosm/kg. Cells were inoculated (passage four) into the fresh basal medium, with differing osmolality, at a VCD of 2.2 x 10^5 cells/mL. The cells were grown in 500 mL shake flasks with a working volume of 325 mL. Operating conditions for the incubator were: 115 rpm, 37°C, 80% rh, and 5% CO_2.

The data for growth, glucose uptake, and lactate production rates for the batch phase portion of the fed-batch experiment (first 72 hours) was presented in Section 4 as Passage 4. On Day 3 (~72 hours), the cultures from passage 4 were fed a bolus of the HGS proprietary nutrient rich feed. The cultures were then fed additional boluses of the proprietary nutrient rich feeds on Day 5 and 7. Concentrated glucose (400 g/L) was added to the culture on days 8 through 11, when required, to bring the glucose concentration to 2 g/L.

Samples were taken daily for VCD, viability, glucose and lactate concentration, pH, and osmolality. Samples retains were saved at -80C for protein quantification by HPLC.

Specific productivity, q_{Ab}, was calculated using the integral method:

$$q_{Ab} = \frac{[mAb]}{\int_0^t x_v dt} \quad \text{Eq. 1}$$

where x_v is the viable cell density concentration and mAb is the concentration of antibody.

5.3 Results

The cultures grown at 290 and 320 mosm/kg produced the highest VCD (Figure 5-1), while culture grown at 450 mosm/kg had the lowest VCD. This was to be expected based on the effect of medium osmolality on growth rate during batch phase experiments (Table 4-1).

The viability of all the cultures was comparable up to 192 hours (day 8) (Figure 5-2). After 200 hours, the viability of the cultures grown at 450 mosm/kg remained high, while the viability of the lower osmolality cultures began to decrease. Due to the higher viability in the 450 mosm/kg culture, the fed-batch experiment was continued for an additional 24 hours. Higher viabilities in cultures grown at 450 mosm/kg were also seen in additional fed-batch experiments (data not presented).

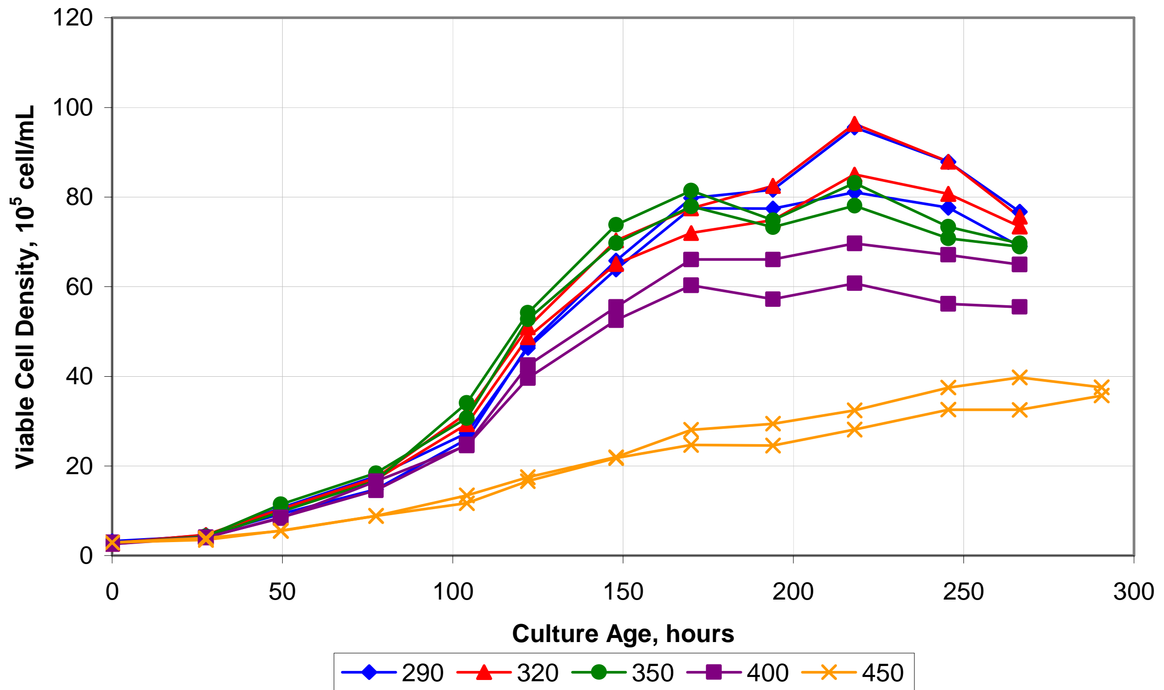


Figure 5-1: Osmolality effect on VCD during the fed-batch process

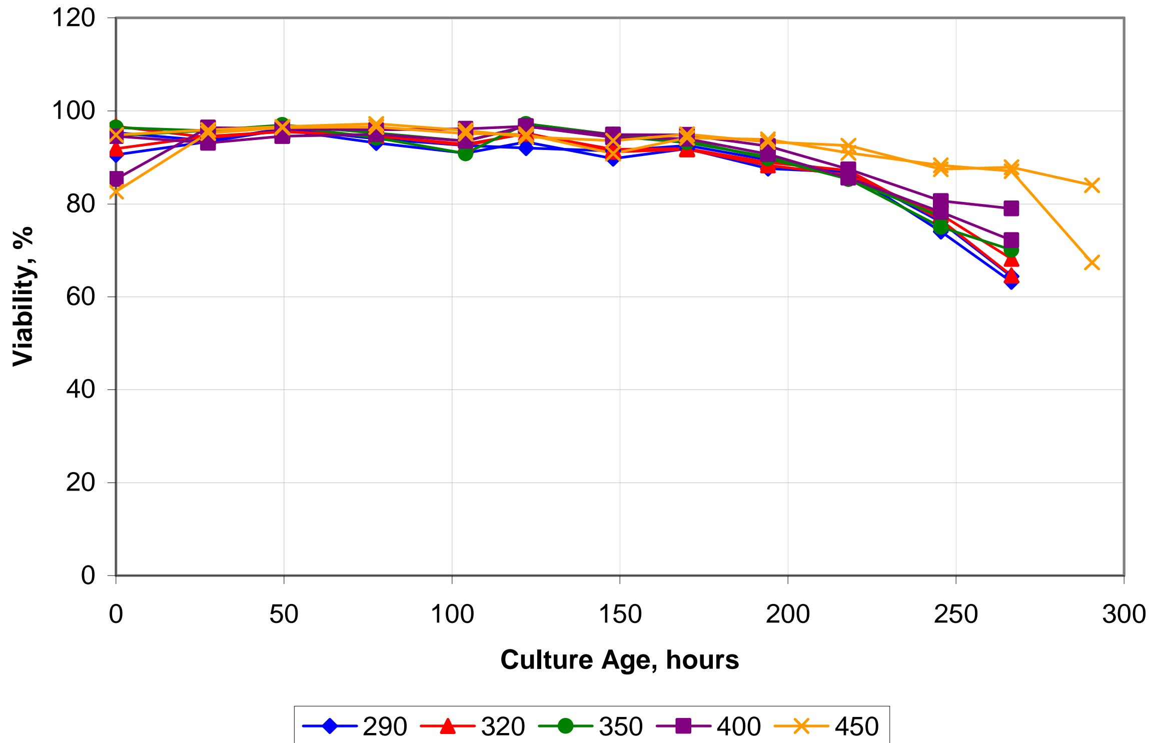


Figure 5-2: Osmolality effect on viability during the fed-batch process

The glucose trends for all the osmotic conditions are similar during the batch phase (up to 72 hours), as the osmolality of the culture increased, so did the glucose consumption rate (Figure 5-3). After the first feed at 72 hours, the glucose trends for the cultures grown at 290 to 400 mosm/kg followed the same pattern. However, after the first bolus nutrient feed at 72 hours, the glucose metabolism for the cultures grown at 450 mosm/kg changed. The glucose consumption rate for the 450 mosm/kg cultures leveled off between 72 and 122 hours, while the consumption rates for the other cultures remained similar to that during the batch phase. This phenomenon was seen again between the second and third bolus feedings.

The lactate trends during the batch phase (up to 72 hours) showed the expected response to the culture osmolality (Figure 5-4). However, the trend for the 450 mosm/kg culture deviated from the other trends after the first nutrient bolus feed. The cultures grown at 450 mosm/kg began to consume lactate after receiving the nutrient rich feed. This phenomenon was not noticed during the previous batch phase passages that were grown out to 88 hours (Figure 4-4 c.). The drop in lactate levels for the other cultures corresponded to an increase in glucose concentration, thus less lactate was being made at this time. This lactate trend, at an osmolality of 450 mosm/kg, was seen in additional experiments (data not published).

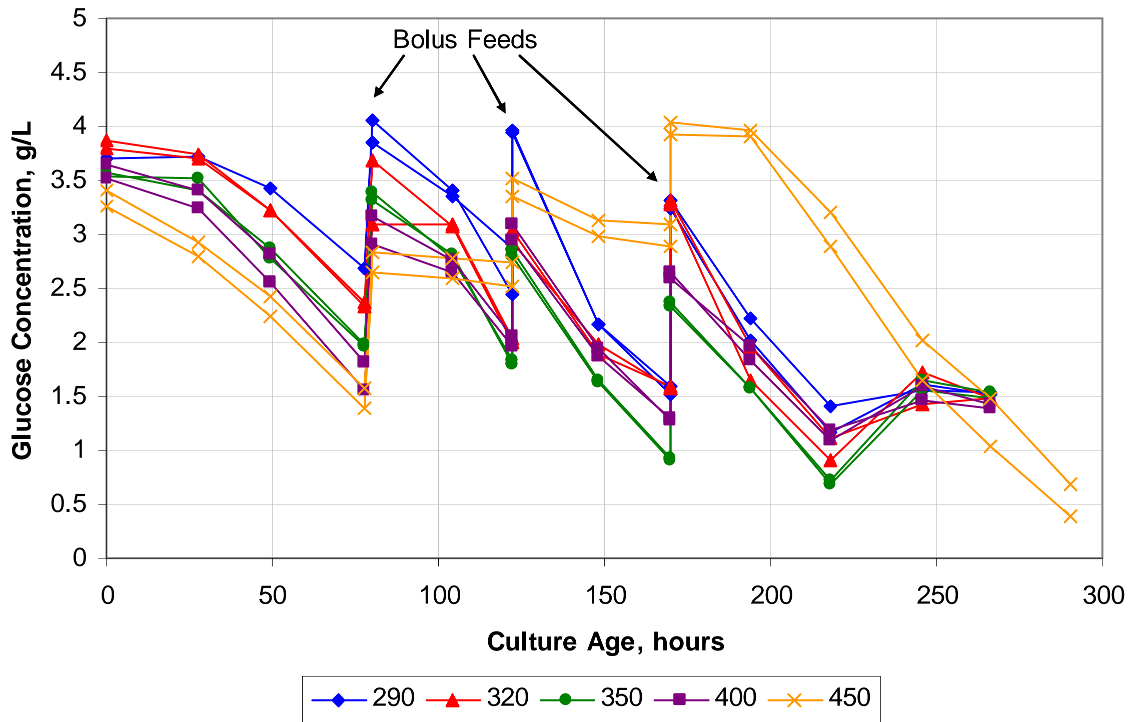


Figure 5-3: Osmolality effect on glucose concentration during the fed-batch process

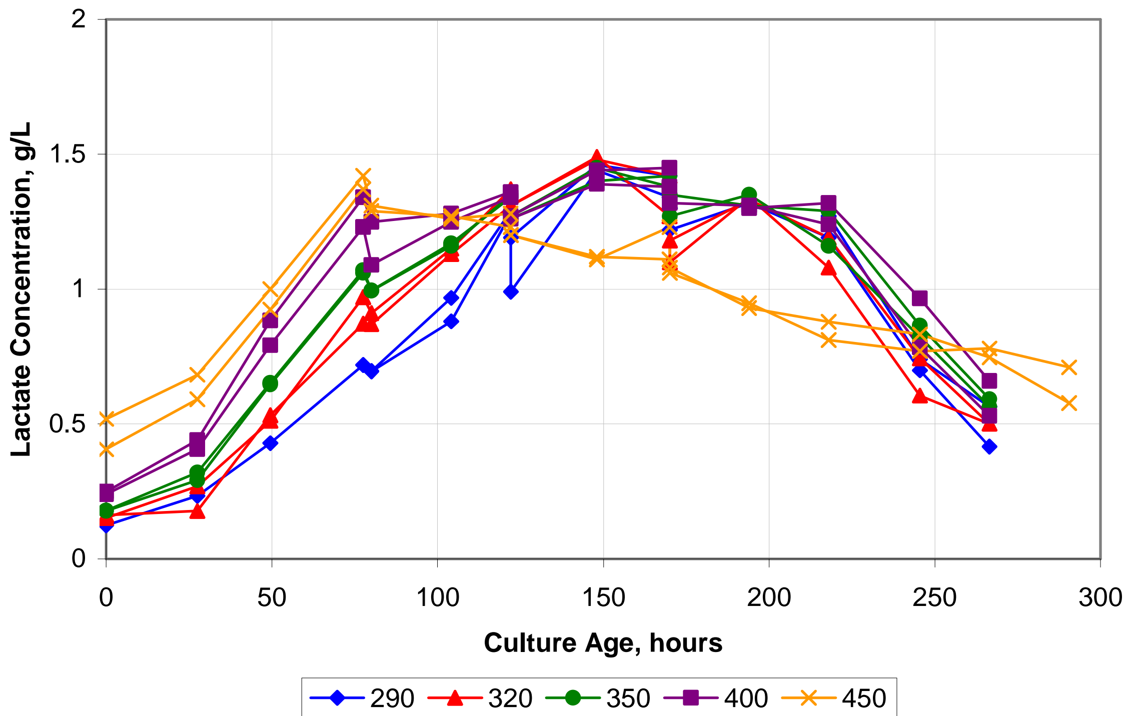


Figure 5-4: Osmolality effect on lactate concentration during the fed-batch process

The pH of the culture medium is greatly affected by the lactate concentration (Figure 5-5). The cultures that produced the greatest amounts of lactate were found to have the largest drop in pH throughout the process. The pH in the 450 mosm/kg culture began to increase once the lactate concentration began to decrease.

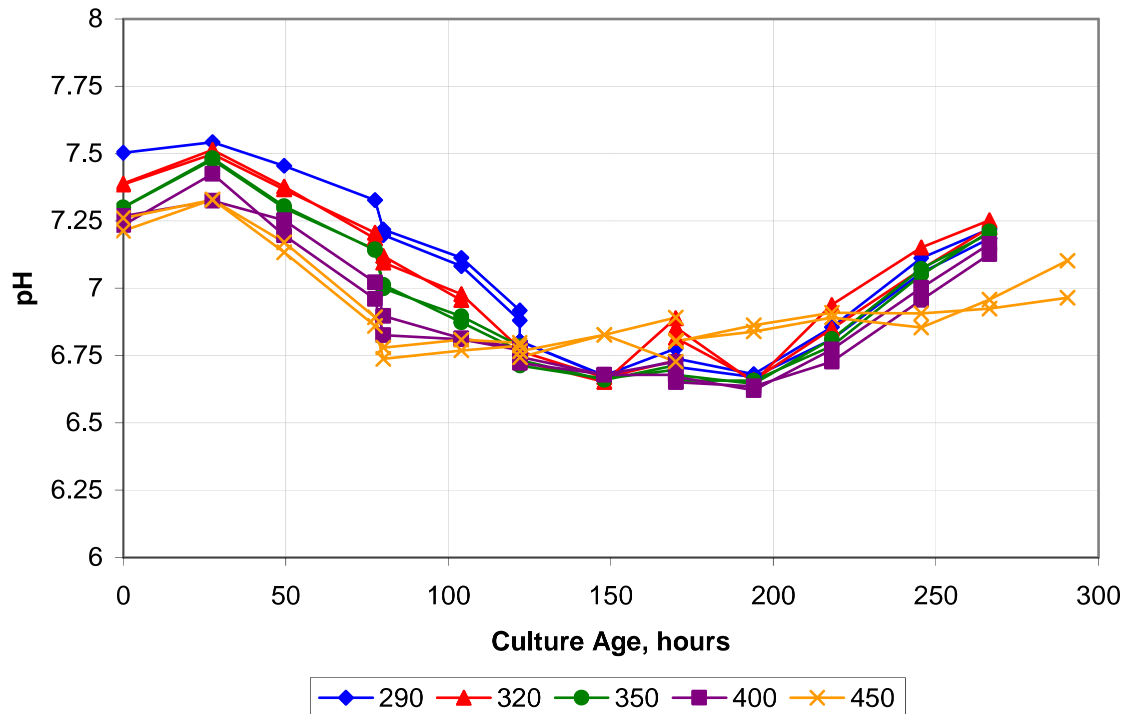


Figure 5-5: Extra-cellular pH level during the fed-batch process

Cells cultured at 450 mosm/kg were found to have a higher cell diameter throughout the fed-batch process. This data is similar to that presented in Figure 4-7 for the batch cultures. The initial drop in cell diameter after exposure to the hyper-osmotic condition was not witnessed during this experiment since the cultures were previously passaged in the different osmolality medium prior to starting to acclimate the cultures.

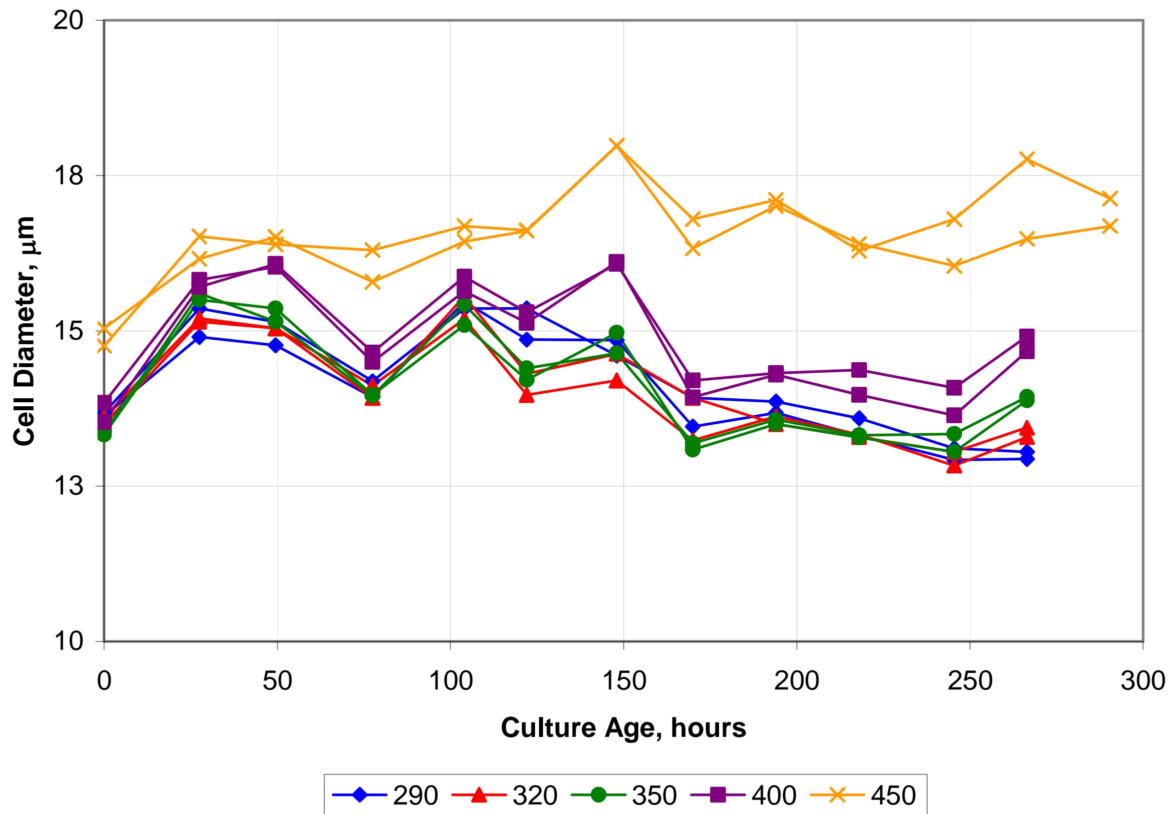


Figure 5-6: Osmolality effect on cell diameter during the fed-batch process

The specific productivity was found to increase with an increase in osmolality (Table 5-2). The 450 mosm/kg cultures had a 142% increase in productivity over the cultures grown at 290 mosm/kg. The increase in osmolality, up to 400 mosm/kg, had a positive impact on the mAb concentration as well. The 450 mosm/kg cultures saw a drop in mAb concentration on Day 11, with the concentration equaling that of the 290 mosm/kg cultures. This culture was extended an extra day due to the high viability. The mAb concentration on Day 12 was 18% higher than on Day 11.

Table 5-2: Effect of osmolality on the average specific productivity[1]

Osmolality, mosm/kg	Relative Average q_{Ab} [1, 2]	Relative Average Day 11 mAb Concentration[1]
290	1.00	1.00
320[3]	1.03	1.04
350	1.12	1.12
400	1.52	1.24
450	2.43	1.00 (1.18 Day 12)

[1] The values presented in the table are relative to the value at the 290 mosm/kg condition.
[2] Value obtained from averaging the q_{Ab} for the duplicate flasks.
[3] Taken from one flask as the second flask became contaminated.

5.4 Discussion

Osmolality Effect on Growth and Viability

The osmolality greatly affected the maximum VCD achieved in the cultures, as the cultures grown at hyper-osmotic conditions were found to produce the lowest maximum VCD (Figure 5-1). These findings correlate with data presented by Wu (2004), who showed that an increase in medium osmolality negatively impacts the growth rate for a GS-NS0 cell line.

The viability of the cultures for the fed-batch experiment was not affected by the increase in osmolality (Figure 5-2). This was also shown during four passages in the batch phase experiments in Chapter 5 (Figure 4-2). At 200 hours, the viability of the cultures grown at 450 mosm/kg remained high, while the viability began to decrease in all the remaining cultures (Figure 5-2). The decrease in viability was possibly due to exhaustion of a non-measured nutrient (i.e. vitamin, amino acid, or trace element). All the cultures received a bolus of the nutrient rich proprietary feed at 170 hours (Day 7). Since the 450 mosm/kg cultures were growing much slower than the other

cultures, it is possible that the nutrients were not exhausted, which allowed the viability of the culture to remain higher than the other cultures.

The osmolality was shown to impact the cell diameter during the fed-batch process as well (Figure 4-7). Cells cultured at 450 mosm/kg were found to have a higher cell diameter throughout the fed-batch process. This data is similar to that presented in Figure 4-7 for the batch cultures and previous data from Ozturk, 1991.

Osmolality Effect on Metabolism

After the addition of the first bolus feed at 24 hours, the glucose and lactate trends for the 450 mosm/kg cultures experienced a different response from the other cultures. The glucose consumption rate decreased between the feed additions due to the slower growth rate (Figure 5-3) and the lactate concentration began to decrease in the culture, indicating that the cells were up taking lactate (Figure 5-4).

Lactate is co-transported across the cell membrane with H^+ ions through monocarboxylic transporters (MCTs). Lactate transport is bidirectional and the lactate re-enters the cell accompanied with an H^+ ion. Lactate transport is highly pH sensitive and the uptake rate is faster when the external pH is lower (Hertz, 2004). Figure 5-5 showed that the external pH of the 450 mosm/kg cell culture was at its lowest (approximately 6.75) around the time the lactate level started to decrease. Due to the lower pH level lactate uptake could have been stimulated.

Once the lactate has entered the cell, it can have multiple uses such as: 1. a substrate for the regeneration of glucose through the gluconeogenesis pathways, 2. conversion to pyruvate and enter the citric acid cycle becoming a precursor for amino

acid or fatty acid synthesis pathways, or 3. formation of osmolytes in response to RVI.

In the absence of glucose, cells can utilize lactate as a carbon source. Glucose can be synthesized from lactate through the gluconeogensis cycle, with lactate being first converted back to pyruvate by lactate dehydrogenase. However, there was clearly enough glucose present in the culture, thus the cells were not requiring lactate to regenerate depleted glucose supplies. Also, proteomic analysis of a CHO cell line in response to hyper-osmotic pressure by Lee (2003), showed that hyper-osmotic conditions promoted an up regulation of pyruvate kinase and glyceraldehydes-3-phospate dehydrogenase. Both of these enzyme drive glycolysis in the forward direction, thus the cell would not be exerting energy to reverse this process.

The second fate of lactate in a cell is the conversion to pyruvate and entry into the citric acid cycle. The citric acid cycle converts pyruvate into a myriad of intermediary compounds needed for the generation of amino acids or it can be converted to citrate and become a building block for fatty acid synthesis. The cell cultures were all fed a proprietary nutrient rich feed, on day 3, 5, and 7 of the fed-batch experiment, which contained protein hydrolysates that provide a surplus of amino acids. Spent medium analysis showed that amino acid levels in all the cultures did not differ due to medium osmolality (data not presented). However, metabolic profiling of the cultures (Chapter 7) showed an increase in internal amino acid concentrations with increasing osmolality. This indicates that the cultures do require either the uptake or generation of amino acids. While Figure 7-2 shows that the increase in internal proline levels was seen in all cultures grown under osmotic pressure, the uptake of lactate was only

observed in the cultures grown at the highest osmotic pressure, 450 mosm/kg. Thus, the demand for amino acid generation could be higher in the hyper-osmotic cultures, requiring the uptake of lactate.

Lactate can also be a precursor for the generation of fatty acids and subsequently lipids (Patel, 1971). Fatty acids are required for numerous functions in the cell. Fatty acids are used in the building of the phospholipids bilayer of the cell membrane. Fatty acids are also essential for protein transport and cell signaling.

The 450 mosm/kg culture had a much larger cell diameter than the other cultures (Figure 5-6). The increased cell diameter would require the cell to need larger concentrations of phospholipids cell membrane than the cells grown at the lower osmotic media. The uptake of lactate in the 450 mosm/kg cultures could be attributed to the larger cell diameter. Previous data by Baquet (1991) suggested a stimulatory effect on cell swelling and lipid generation. However, the metabolic profiling performed on these cultures did not see a response to fatty acid concentrations and osmotic pressure (Chapter 7).

Lastly, cells introduced to increase osmolality experience RVI. The cell accumulates electrolytes and osmolytes in order to regulate the cell volume after the initial decrease in size due to osmotic pressure. Since electrolytes alter ion gradients and can compromise cell function, cells produce osmolytes which can alter the osmolality, and subsequently cell volume, without inhibiting cell function (Lang, 1998 a.). Mammalian cells produce three types of osmolytes: polyalcohols, methylamines, and amino acids and their derivatives. The formation of methylamines, such as

glycerophosphorylcholine require the addition of fatty acids, thus generation of increased fatty acids would be required under hyper-osmotic conditions.

Osmolality Effect on mAb Production

The 450 mosm/kg culture also had a higher specific productivity than the other cultures, 60% higher than the cultures grown at 400 mosm/kg and 140% higher than the cultures grown at 290 and 320 mosm/kg (Table 5-2). Proteomic analysis of GS-NS0 cell cultures with varying mAb production rates was shown to have an upregulation of chaperone proteins required for protein synthesis, such as GRP78 and PDI (Alete, 2005). Alete (2005) also showed an upregulation of POR1 and POR2, porin proteins required for the formation of channels in the lipid bilayer for the transport of hydrophilic molecules. An increase in pore formation as well as an increase in chaperone proteins would require an increased need of fatty acids and lipids. Thus, the uptake of lactate in the 450 mosm/kg could be due to increased requirements of fatty acids due to the higher mAb specific productivity.

The hyper-osmotic pressure did have a positive impact on the specific productivity of the cell line and these results correlate with the findings by Wu (2004) on a similar GS-NS0 cell line. The 450 mosm/kg cultures did have the highest q_{Ab}, however, the decrease in growth rate lead to lower accumulation of viable cells and thus lower overall mAb concentration (Table 5-2). This result is similar to findings by Ozturk (1991), Wu (2004), Oyaas (1994), and Lin (1999 b.), on a number of different cell lines. The mAb concentration was found to increase with increasing osmolality up to the 400 mosm/kg condition. For this cell line there appears to be a threshold for overall mAb produced between 400 and 450 mosm/kg.

Chapter 6 Effect of an Osmolality Shift on Monoclonal Antibody Production

6.1 Introduction

Many industrial processes utilize a shift in a process parameter to stimulate mAb production. A shift in temperature during mAb production has shown to maximize productivity in CHO cell lines (Fox, 2004; Kauffmann, 1998), while the response of a GS-NS0 cell line to pH shifts has been studied by Osman (2001). To employ a process shift, the cell culture is first grown at one set of conditions which maximize cell growth rate. Once the VCD of the culture is high, process parameters are adjusted to values that are conducive for high mAb production rates.

The previous experiments (Chapters 5 and 6) have shown that the GS-NS0 cell line has a higher growth rate at lower osmolality medium and higher mAb productivity at higher osmolality levels. Thus, it has been hypothesized whether a shift in medium osmolality could be beneficial for increased mAb production. To test this hypothesis, cultures were grown at three different low osmolality conditions (290, 320, and 350 mosm/kg) which were shown to promote high growth rates for this cell line (Chapter 5). The osmolality of the cultures was then shifted to 450 mosm/kg on either day 5 or 7 of the process in order to stimulate mAb production.

6.2 Methods

Basal medium was prepared at 290, 320, and 350 mosm/kg, by adjusting the concentration of NaCl in the medium. Synthetic cholesterol was added to the basal medium.

A GS-NS0 cell culture, initially grown at approximately 300 mosm/kg, was obtained for use as an inoculum for this experiment. The inoculum culture was added to each medium (290, 320, or 350 mosm/kg) at a starting VCD of 2.2×10^5 cells/mL. The cultures were passaged into the respective medium three times before performing the fed-batch production experiment. This was performed to acclimate the cells to the different osmolalities. The fed-batch experiment started on the fourth passage. The osmotic shift conditions for this experiment are illustrated in Table 6-1.

Table 6-1. Growth conditions for the osmolality shift experiments

Shake Flask Number	Growth Medium Osmolality, mosm/kg	Osmotic Shift / Day
1 and 2	290	None
3 and 4	290	Day 5
5 and 6	290	Day 7
7 and 8	320	None
9 and 10	320	Day 5
11 and 12	320	Day 7
13 and 14	350	None
15 and 16	350	Day 5
17 and 18	350	Day 7

For the osmotic shift experiment, shake flasks were inoculated to a starting VCD of 2.2×10^5 cells/mL. On Days 3, 5, and 7 each shake flask received a bolus addition of the proprietary nutrient rich feed. On Day 5 or 7, after the addition of the feed, the

osmolality of the shake flasks shown in Table 6-1 was adjusted to 450 mosm/kg by the addition of concentrated NaCl. The cultures were all given a concentrated glucose feed to increase the glucose concentration to 2 g/L, if required, on Days 8 through 10. Note: some shake flasks did not receive a shift in osmolality and were maintained as control flasks for that osmotic condition.

All the experiments were performed 250 mL shake flasks with a working volume of 125 mL. Operating conditions for the incubator were: 115 rpm for Day 0 – 3 and 160 rpm for Day 3 - 11, 37°C, 80% relative humidity (rh), and 5% CO_2. The shaker agitation rate was increased after Day 3 to ensure adequate oxygenation to the cell cultures. Each condition was performed in duplicate.

Samples were taken daily to measure VCD, viability, glucose and lactate concentration, pH, and osmolality of the cultures. The time course data was used to calculate the following metabolic parameters during exponential growth: glucose consumption rate, lactate production rate, and cellular growth rate.

6.3 Results

The osmotic shift to 450 mosm/kg had a great impact on the maximum VCD achieved in the experiment (Figure 6-1). The cultures with the osmotic shift on Day 5 produced the lowest maximum VCD, while the cultures that did not experience an osmotic shift were capable of producing the highest maximum VCD. Note: the VCD values in Figure 6-1 are the average values for the duplicate shake flasks.

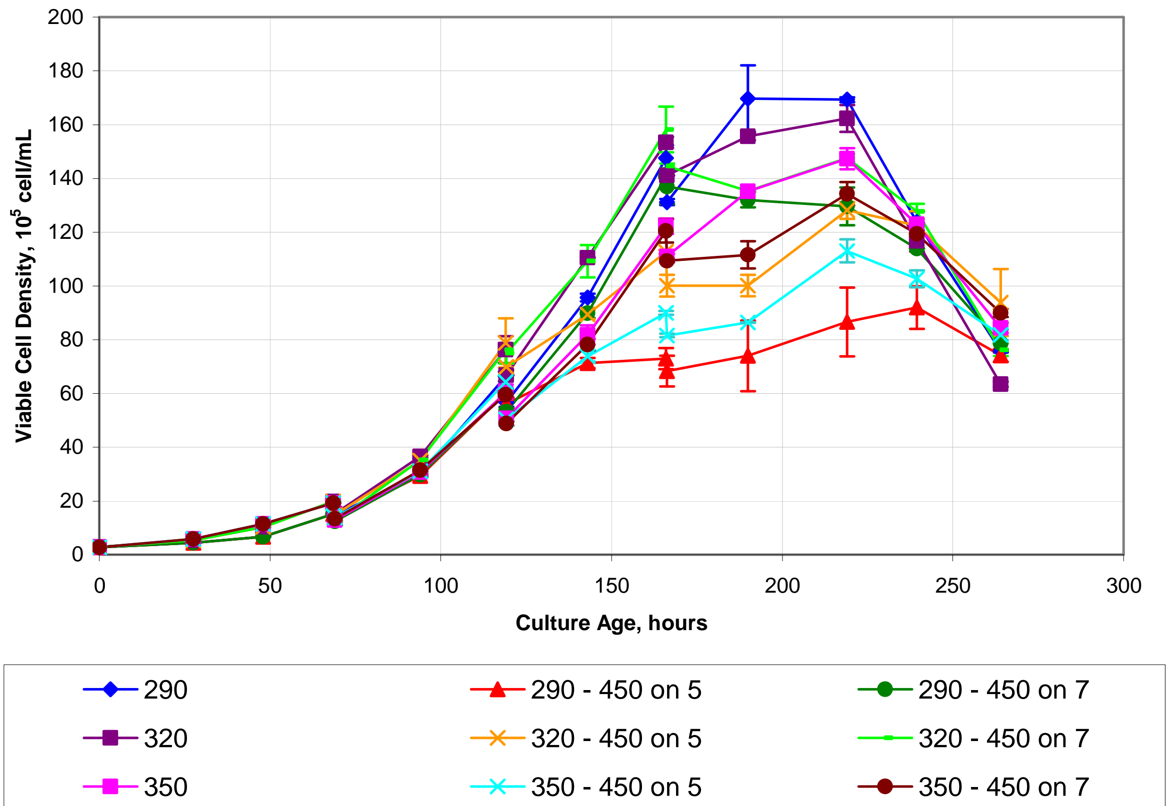


Figure 6-1: Osmolality shift effect on average VCD

The viability of the cultures dropped after the shift in medium osmolality. The 290 mosm/kg cultures which had the greatest increase in osmolality on Day 5 were found to have the biggest drop in viability due to the osmotic shift (Figure 6-2). Overall, the cultures that did have an osmotic shift had the highest viability at the end of the process. Note: the viability values in Figure 6-2 are the average values for the duplicate shake flasks.

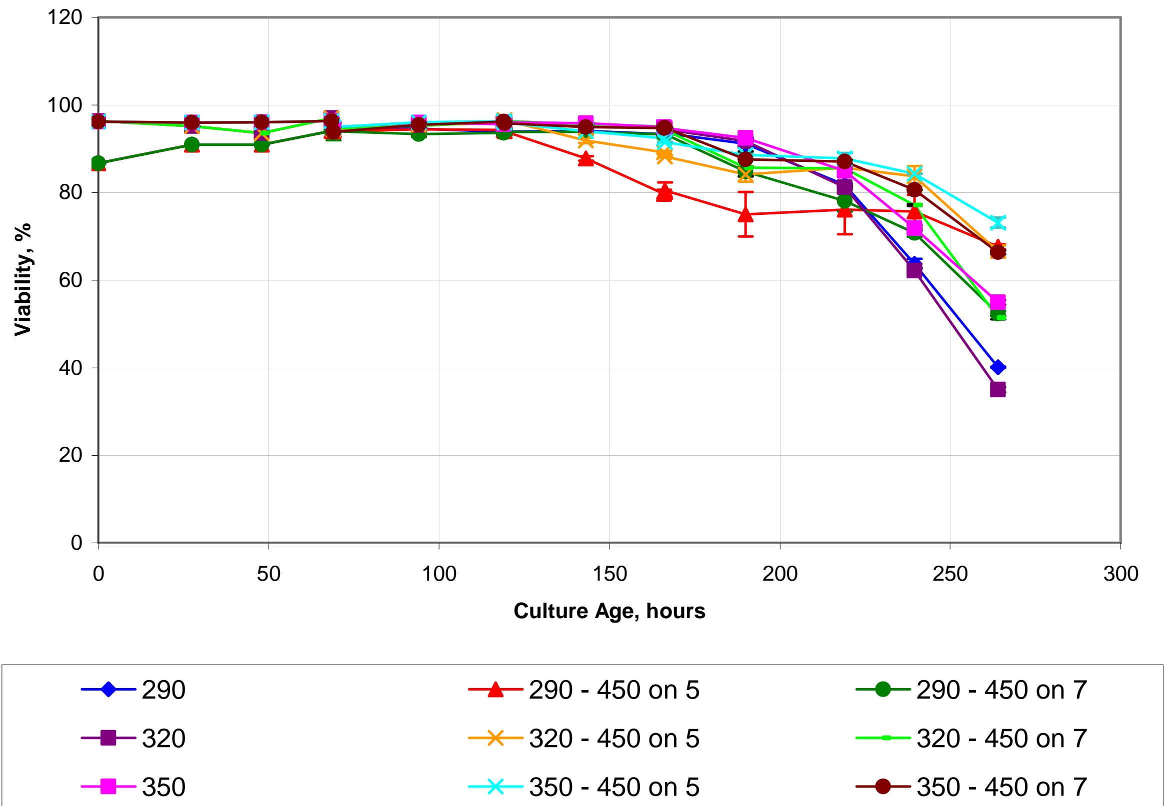


Figure 6-2: Osmolality shift effect on average viability

The glucose (Figure 6-3) and lactate (Figure 6-4) concentrations showed a response to the medium osmolality. During the batch phase (up to Day 3 / 72 hours), the cultures grown at 350 mosm/kg had the largest drop in glucose concentration and the largest production of lactate. During the fed-batch phase, the glucose concentration was fairly consistent between conditions, except for the 290 → 450 on Day 5, which showed a slower consumption of glucose due to the reduced cell mass. These cultures also had the highest lactate concentration, as expected since they received the largest change in osmolality. The lactate concentration during the fed-batch phase showed great variability and concentrations cannot be correlated with a particular conditions based on this limited set of data.

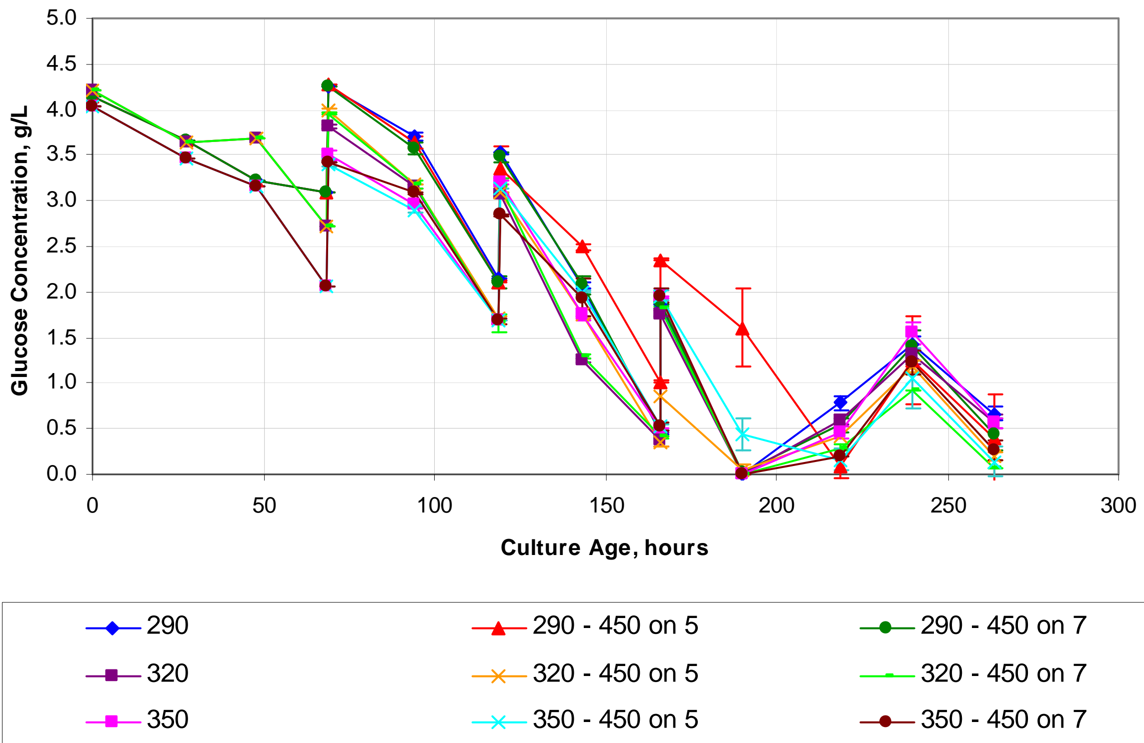


Figure 6-3: Average glucose trends for osmolality shift experiments

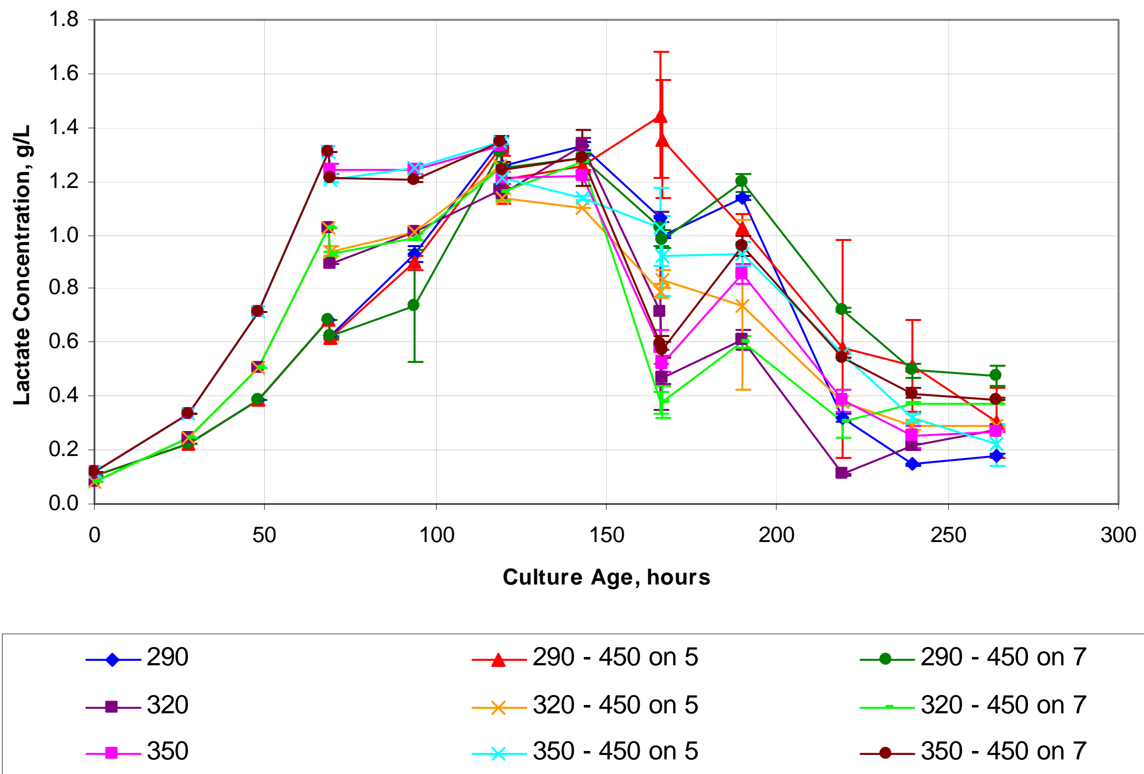


Figure 6-4: Lactate trends for osmolality shift experiments

The mAb concentration correlated with osmolality, as the shake flasks that did not receive an osmotic shift had increased final mAb concentration with increased medium osmolality (Table 6-2). The osmotic shift was beneficial in increasing specific productivity and the cultures that received the shift in osmolality on Day 5 as opposed to Day 7 had the largest increase in qAb (Table 6-2). The 350 → 450 on Day 5 cultures produced the highest mAb concentration. Note: the mAb concentration reported in Table 6-2 is the average concentration for the duplicate shake flasks.

Table 6-2: Average specific productivity and final titer for the osmolality shift experiments[1]

Osmolality Condition, mosm/kg	Relative Average q_{Ab}[1]	q_{Ab} Change due to Osmotic Shift, %	Relative Average Final Titer[1]	Titer Change due to Osmotic Shift, %
290	1.00	NA	1.00	NA
290 → 450 on Day 5	1.57	57.2	1.00	No Change
290 → 450 on Day 7	1.19	19.3	1.02	1.89
320	1.08	NA	1.10	NA
320 → 450 on Day 5	1.41	32.7	1.21	9.4
320 → 450 on Day 7	1.20	11.2	1.22	10.3
350	1.29	NA	1.16	NA
350 → 450 on Day 5	1.74	34.1	1.29	11.4
350 → 450 on Day 7	1.59	23.1	1.25	8.1

[1] The value reported are relative to the value determined for the 290 mosm/kg condition.
[2] The final mAb concentration and q_{Ab} values were averaged for the duplicate shake flasks.

6.4 Discussion

The osmotic shift was shown to reduce the maximum VCD of the cultures (Figure 6-1). This result was to be expected based on the growth rates calculated for the cell line in batch phase experiments (Table 4-1). The cells grown at 450 mosm/kg were shown to have upwards of a 30% increase in growth rate over the cultures grown at the lower osmotic conditions.

The osmotic shift also caused a considerable viability drop in the cultures, mainly the cultures grown at 290 mosm/kg (Figure 6-2). However, the cultures that received the osmotic shift had the highest viability at the end of the process. This result was also seen in the fed-batch experiments (Figure 5-2). The 450 mosm/kg culture was still over 80% viable on Day 11. The slower growth rate caused by the osmotic shift can contribute to the higher viabilities of these cultures. It is possible that a non-measured nutrient had exhausted in the cultures with the higher viable cell densities, apparent from the crash in viability, beginning on Day 10, for the cultures that did not receive an osmotic shift (Figure 6-2).

The purpose of these experiments was to test if an osmotic shift would positively impact productivity and this was found to be true. The cultures grown at 350 mosm/kg, which received the osmotic shift, were found to be the highest producers of mAb (Table 6-2). Based on the previous data from the batch and fed-batch experiments, this finding could have been predicted. The batch phase experiments (Table 4-2) showed that the optimum osmolality for cell growth was 350 mosm/kg and the fed-batch experiments showed that the optimum osmolality for protein production was 450 mosm/kg (Table 5-2). Thus, the cells were grown in the most conducive

osmolality condition for growth prior to shifting to the most conducive condition for protein production.

The timing of the osmotic shift was found to impact the productivity and mAb concentration. The cultures which received the shift in osmolality on Day 5 as opposed to Day 7 had a higher increase in both q_{Ab} and mAb concentration (Table 6-2). Further optimization into the timing of the osmotic shift could prove to be beneficial to protein production.

Chapter 7 Metabolic Analysis of Osmolality Effect on NS0 Cells

7.1 Introduction

As stated in section 2.3, the application of metabolomics to mammalian cell culture has not been widely explored. Techniques used to quench the metabolism of microbial and yeast cell lines, might not be sufficient to stop the metabolism of mammalian cell cultures. Errors in sampling and quenching can lead to an inaccurate representation of the metabolic profile of these cell lines. Advances such as a micro heat exchanger to rapidly sample and quench mammalian cells are currently being developed (Wiendahl, 2007).

In this work, quenching cells by snap-freezing in liquid nitrogen was used. This method is similar to that used by Griffin (2000), *et al.* in their work with rodent cell culture samples. Extraction was performed using a methanol/water method previously developed for potato tuber cultures (Roessner, 2000). Derivatization of the polar metabolites was performed using methods described by Dutta (2009) for *Arabidopsis thalia*. Identification and quantification of the derivatized metabolites was performed using a GC-MS method and data analysis method previously described by Kanani and Klapa (2007).

The metabolomics methods referenced above were evaluated for use on the GS-NS0 cell line. Finger printing of the GS-NS0 metabolic response to osmolality was determined.

7.2 Methods

Quenching, Extraction, and Derivatization

Cells were removed from the shake flask experiments performed in Chapters 4 (81 hours, Passage 3 / Day 4) and 6 (72 hours, Passage 4 / Day 3 and 144 hours, Passage 4 / Day 6) and were washed and centrifuged (Sorvall Model, 1000 g for 5 minutes) in ice cold phosphate buffer (pH 7.0) to remove the spent medium and external metabolites. After washing, the cell pellets were snap frozen in an ethanol / dry ice bath to quench metabolism and remained in the bath during cell lysis manipulations. A 0.2 mg of ribitol per gram of cell mass was added to each sample (ribitol was used as an internal standard for the extraction and derivatization assay). Cold methanol stored in dry ice (28 mL / g of cells) was added to each sample to extract metabolites while the cells were homogenized using a tissue grinder.

The homogenized cells in the methanol mixture were heated to 70°C for 15 minutes in a water bath. After heating, water was added to each sample and the suspension was centrifuged at 4000 g for 10 minutes. The supernatant containing the internal metabolites was removed and aliquoted into glass tubes. The metabolite samples were then vacuum dried over night at room temperature. After drying, the samples were stored at -20°C until derivatization.

The metabolites were converted to their methoxime trimethyl-silyl derivatives. The derivatization was carried out in two steps. First 100 µL of methoxyamine hydrochloride solution in pyridine (20 mg/mL) was added to each of the dried extracted samples. The samples were incubated at room temperature for 120

minutes. Next, 100 µL of silylating agent, MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) was added and the samples were incubated for four hours. Fifty µL of the derivatized metabolite solution were then placed into GC-MS autosampler vials.

Metabolite Identification and Analysis

The samples were analyzed using a Varian 2000 GC-(ion trap) MS system run using a previously established GC-MS metabolomic methods (Kanani and Klapa) (40:1 split ratio, 1 mL/min column flow rate, and 3 scans per sec scanning rate using 1 µL of the injected derivatized solution). Optimization of the split ratio was performed to ensure all peaks, except phosphate, were below the saturation region and within the linear operating range of the instrument. A high concentration of phosphate was present in the samples due to residual phosphate buffer used for washing the cells when removing the extracellular metabolites. The GC-MS temperature program was as follows: hold 70°C for 5 min, 5°/min heating ramp up to 320°C, and hold for 1 min at 320°C; thus the total run time of the GC-MS run was about 55-57 minutes. Two instrument replicates were run for each biological sample.

The raw data obtained from GC-MS analysis was processed using Varian instrument software and data processing methods for peak quantification developed by researchers at University of Maryland, College Park (UMCP). Peaks were identified by using (1) an in house library of standard metabolites, (2) NIST mass spectrometry

library, and (3) publicly available GC-MS standard libraries. The raw peak areas obtained for each metabolite were then normalized using a proprietary data correction and normalization algorithm developed by researchers at UMCP (Kanani and Klapa, 2007). Multivariate data analysis was performed on the normalized peak areas using open source freely available Multi Experiment Viewer (MeV) software. Metabolite quantification and data analysis was performed by Harin Kanani under the direction of Dr. Maria Klapa, Chemical and Biomolecular Engineering Department, University of Maryland, College Park.

7.3 Results

Figure 7-1 shows a typical GC-MS profile for a cell culture sample. The x-axis is the retention time and y-axis is the total ion intensity recorded by the mass-spectrometer at the given retention time. In order to overcome the problem of co-eluting peaks, unique m/z and retention time combinations were used for quantification of individual metabolites. 650 metabolite derivative peaks were detected in at least one of the samples, but after filtering, data correction, and normalization only 170 consistent metabolites were used for data analysis. From these, 63 metabolites were with known structure and another 20 metabolites had at least partial classification. The rest of the metabolites could not be identified based on existing databases. Table 7-1 lists the metabolites that were consistently identified in the cell culture samples.

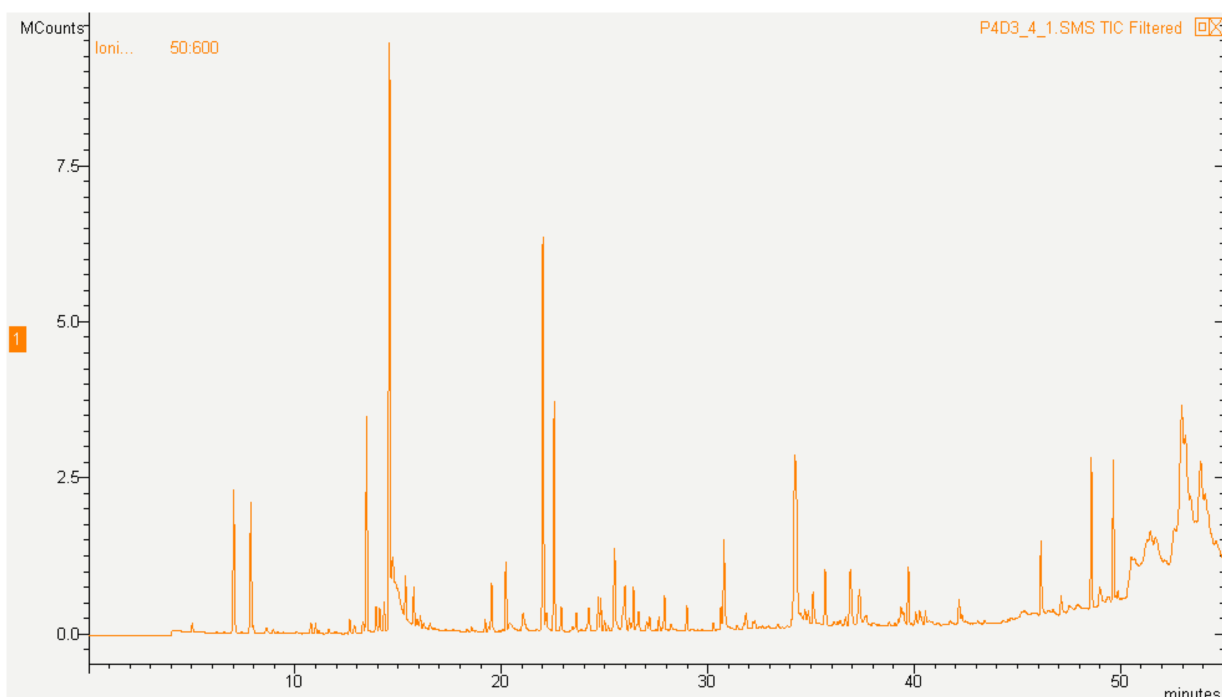


Figure 7-1: A GC-MS metabolomic profile of a typical cell culture sample

Table 7-1: List of consistently known metabolites in the GC-MS metabolomic analysis

Nitrogen Compounds	6-Hydroxynicotinate, Adenoise, Allothreonine, Asparagine, Aspartate, b-Alanine, Cystine, Ethanolamine, Glutamine, Histidine, Isoleucine, Indole, Leucine, Lysine, Methinonine, N-acetylglutamate, Ornithine+Arginine, Phenylalanine, Proline, Serine, Threonine, Uracil
Sugars	Arabinose, Arabinofuranose / Ribofuranose, Erythritol, Fructose, Fructose-6-Phosphate, Glucose, Maltose, myo-Inositol, myo-Inositol-(1 or 2)-phosphate, Rhamnose, Sorbitol, Xylitol
Organic Acids	2-bis-Hydroxybutanoic Acid, 2-hydroxybutanoic Acid, 2-Hydroxyglutarate, 4-Hydroxybutanoic Acid, Citramalate, Citrate, Fumarate, Gluconate, Glycerate, Glycolate, Glyoxilate, Malate, Pyrrole-2-carboxylic Acid, Pyruvate, Succinate, Threonate
Lipid Components	α-Linolenic Acid, Glycerol, Glycerol-2-phosphate, Glycerol-3-phosphate, 3-Phospho-glycerate, Monomethylphosphate, Octadecanoic Acid, Phosphoric Acid
Secondary Metabolites	Ascorbate, Eiconsanoate, Methyl Benzoate, Sinapinic acid, Stigmasterol

Figure 7-2 shows an example of the GC-MS metabolomic data peak identification and relative quantification. The profile is from a sample taken at the end of the batch phase (Passage 3 / Day 4). Proline shows an increase in its concentration with increasing osmolality.

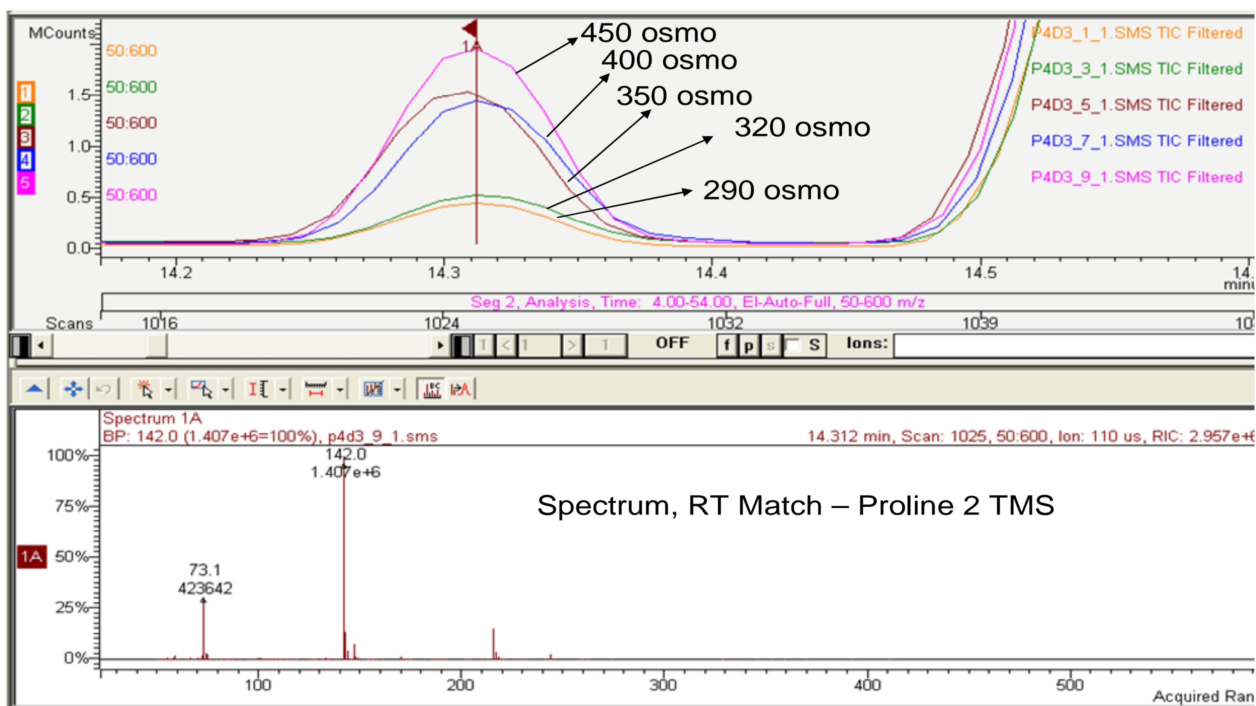


Figure 7-2: Comparison of proline peak under different osmolality conditions

The data was normalized to the metabolomic profile of 290 mosm/kg for each of the three sample times obtained and averaged for two biological and two instrumental replicates. A clear separation between the metabolomic profiles of passage 3 / day 4 (end of batch phase; green), passage 4 / day 3 (end of batch phase; red) and passage 4 / day 6 of mAb production process (blue) profiles is shown in Figure 7-3. This suggests the passage and age of the culture has a strong influence on concentration of metabolites – which is stronger than the effect of different osmolality. Within the three sample groups however, it can be seen that the 400 mosm/kg

clusters with 450 mosm/kg. Thus, there seems to be a significant jump in metabolomic profiles between concentration 350 and 400 mosm/kg.

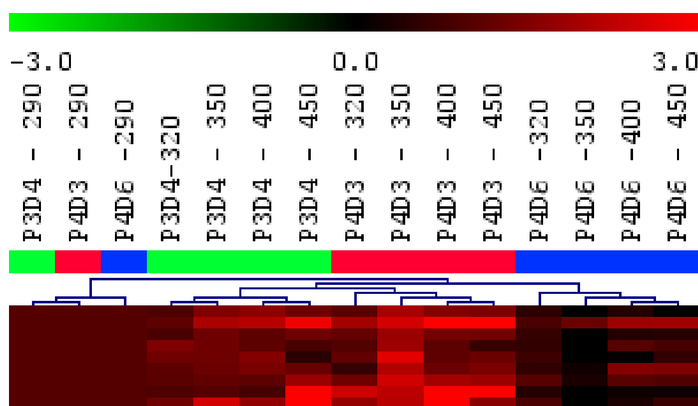


Figure 7-3: Heat map of the hierarchical clustering of average normalized samples

A cluster of 22 metabolites (9 known) were identified, which showed significant increase (up to 50 fold) between the high osmolality and low osmolality conditions. In spite of differences between the passage and age of the culture, these metabolites showed a consistent increase, almost proportional, to the osmolality suggesting a very strong link to the osmotic response of the cultures (Figure 7-4).

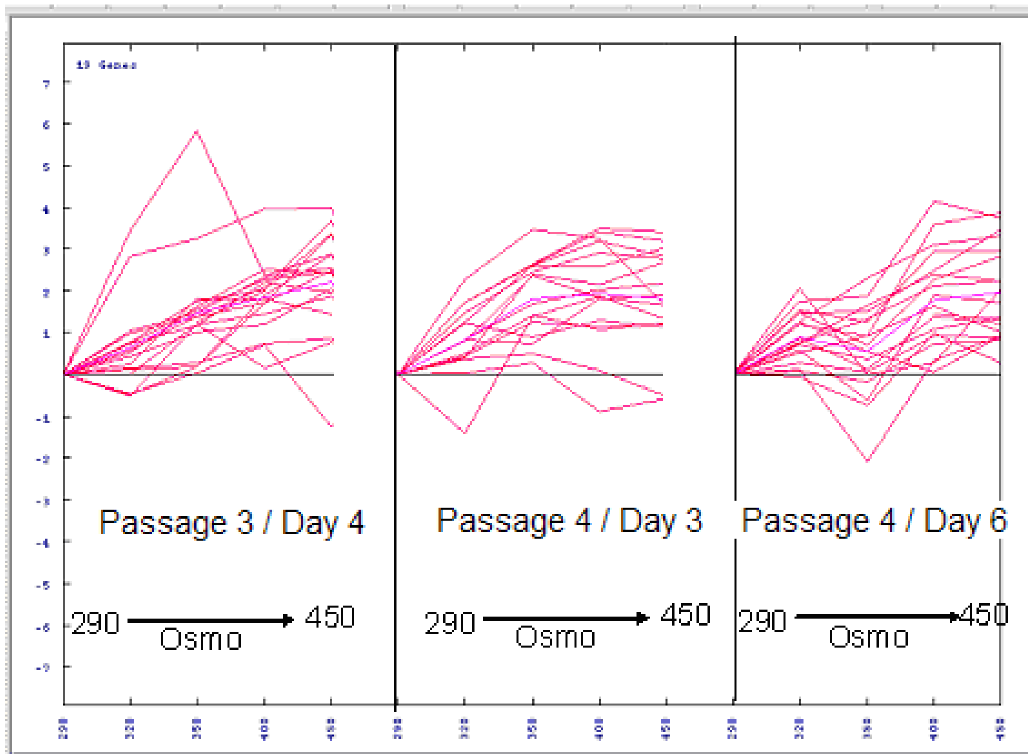


Figure 7-4: K-means clustering of the averaged metabolomic profiles.

A heat map of the cluster of metabolites identified in Figure 7-4 is shown in Figure 7-5. The heat map shows proline, serine, and phenylalanine were all upregulated in hyper-osmotic medium.

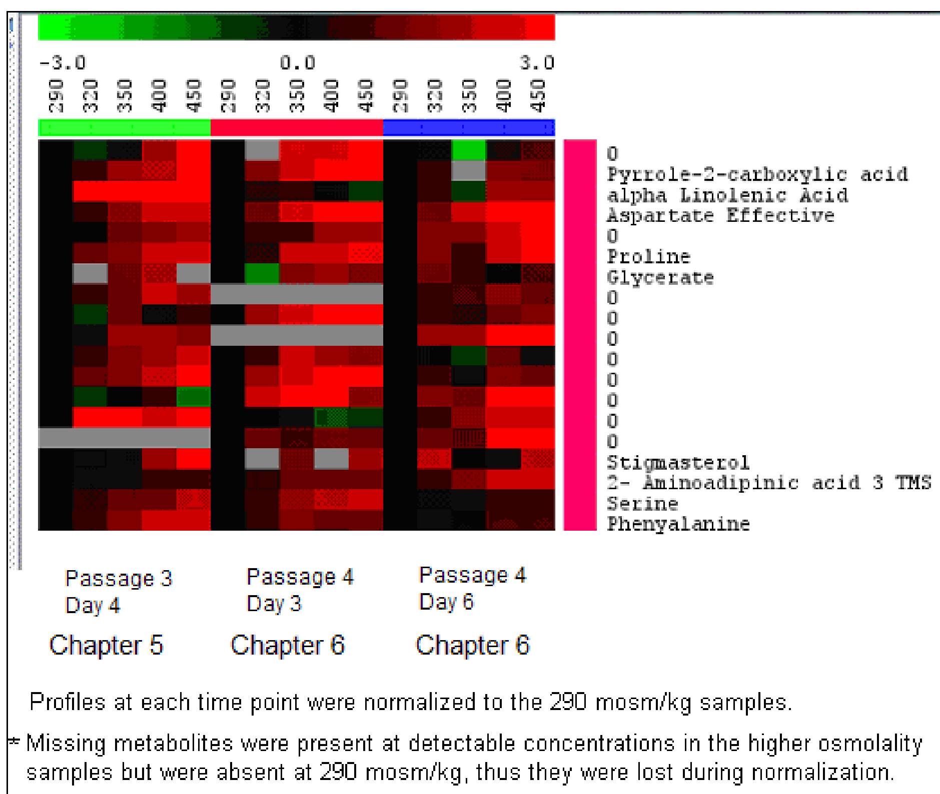


Figure 7-5: Heat map of the metabolites identified from the K-means clustering

7.4 Discussion

Despite using quenching and extraction steps developed for plant cell cultures, a dose response in the NS0 cell culture to medium osmolality was found. Proline concentrations were shown to increase as the osmolality of the medium increased (Figure 7-2). Proline can be used by the cell as an osmolyte in order to return the cell to its original volume when experiencing a regulatory cell volume increase due to hyper-osmotic conditions (Lang 1998 a.) Since the cells grown at 450 mosm/kg were found to be the largest after RVI, an increase in osmolytes, such as proline, would be required.

The cell age or stage of the fed-batch process, was found to have a stronger influence on the metabolite concentration than the medium osmolality (Figure 7-3). This finding was to be expected as the cellular environment differs drastically due to metabolites present in the batch vs. nutrient rich feed medium during a fed-batch process. The basal batch medium is a very basic medium that does not contain a large quantity of amino acids, while the nutrient rich feed contains large concentrations of amino acids and small polypeptides. The passage number also had a strong influence on the metabolite concentration (Figure 7-3). It would be predicted that samples taken at the end of the batch phase in both passage 3 and 4 would have similar concentrations in metabolites. However, the growth, glucose uptake, and lactate production rates were found to differ between passages (Figure 4-3, Figure 4-5, and Figure 4-6). These differences would lead to dissimilar metabolite concentrations.

A cluster of 22 metabolites were found to significantly increase with increasing osmolality (Figure 7-4). Nine of these metabolites were identified including multiple amino acids and their derivatives were present in higher concentrations in the cells grown under hyper-osmotic conditions. As stated above, amino acids and their derivatives are used as osmolytes to help cell's regulate their volume in hyper-osmotic conditions.

Chapter 8 Conclusions

The osmolality of the growth medium was shown to greatly impact the cell growth and metabolism of the GS-NS0 cell line evaluated. Growth medium at 350 mosm/kg was found to be optimum for cellular growth rate, while 450 mosm/kg medium was found to be optimum for specific productivity of the monoclonal antibody. The metabolism was affected by the change in medium osmolality. Glucose uptake and lactate production rates increased with increasing osmolality. In fed-batch cultures grown at 450 mosm/kg, cellular uptake of lactate was witnessed. It is hypothesized that the lactate uptake was due to the increased mAb productivity rate and the increased cell size of these cultures. Lactate can be utilized by the cell in the production of fatty acids. Additional fatty acids would be required by cells with increased protein productivity and increased cell diameter.

The GS-NS0 cell line showed cell volume regulatory mechanisms. When the cells were first introduced to hyper-osmotic medium, the cells initial shrunk as a result of the osmotic pressure gradient created. However, the cells were able to increase their cell size through RVI mechanisms and return to a more normal cell size. RVI requires the formation of osmolytes in the cell in order to alter the osmolality without affected cellular function. Metabolic analysis of the cells found that the common osmolytes, proline, was higher in cells cultured under hyper-osmotic conditions.

The mAb productivity was greatly increased with an increase in medium osmolality. Cells grown at 450 mosm/kg were found to have a 140% increase in productivity over

those grown at 290 or 320 mosm/kg. However, due to lower cellular growth rates, the total mAb produced did not increase at hyper-osmotic conditions. Antibody concentrations were found to be at a maximum in the cultures grown at 400 mosm/kg; at 450 mosm/kg the mAb concentration dropped almost 20%.

An osmotic shift proved to be beneficial in increasing the antibody concentration in the cell culture. Cells initially grown at 350 mosm/kg and then shifted to 450 mosm/kg were found to produce the highest concentration of mAb. The osmotic shift increased q_{Ab} for all conditions evaluated and q_{Ab} was found to be dependent on the timing of the osmotic shift.

The metabolomic analysis of the GS-NS0 cell line showed a dose response in the internal concentration of amino acids and their derivatives. Proline concentrations were found in increasing concentration in the cells grown under hyper-osmotic conditions.

Chapter 9 Future Studies

The research on the response of the GS-NS0 cell line has just scratched the surface. Osmolality has been shown to greatly impact the metabolism of the cell, however little is still known on the mechanisms of its impact. Lactate was found to be up taken into the cell in cultures grown under hyper-osmotic conditions, however, the fate of the lactate is unknown. It has been postulated that lactate could be used for the formation of amino acids, osmolytes, or fatty acids. The metabolic profiling performed on the cultures was inconclusive in determining the fate of lactate. Further analysis using tools such as metabolomics and proteomics can provide a vast insight into the utilization of lactate in osmotically stressed cells. Additional experimentation, such as oil red O staining, could help in determining an increase in fatty acid concentrations on the surface of cells grown under hyper-osmotic conditions.

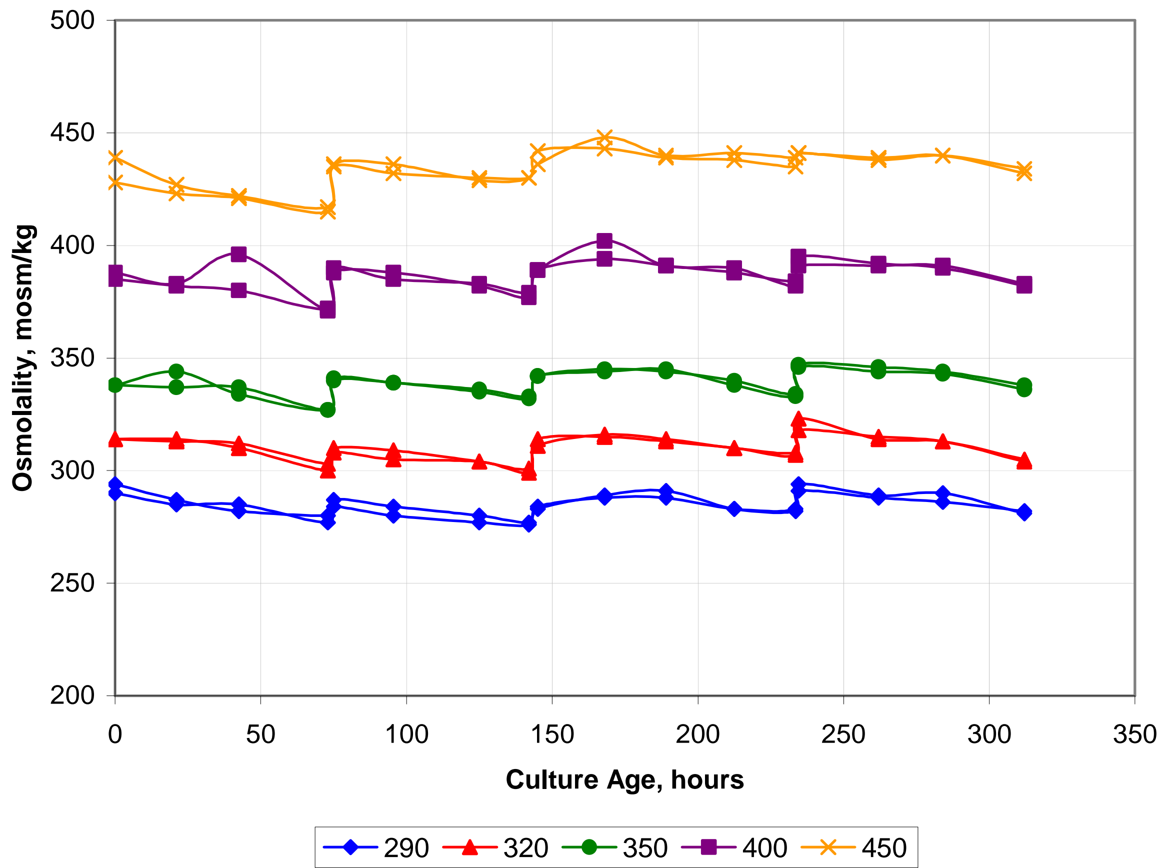
Additionally, cultures grown under hyper-osmotic conditions showed an increase rate of glucose consumption. The increase in glucose uptake was did not contribute to an increase in cell concentration for these cultures. The increase in glucose consumption could be due to a higher energy demand for the culture. A carbon mass balance to quantify the CO_2 formation from the cell culture under different osmotic conditions could be performed to analyze the energy requirements of these cultures.

Furthermore, hyper-osmotic conditions are favorable for the production of monoclonal antibodies. It is still not clearly understood why osmotically stressed cells are better mAb factories. Finding the reasons to how cells can increase their productivity will

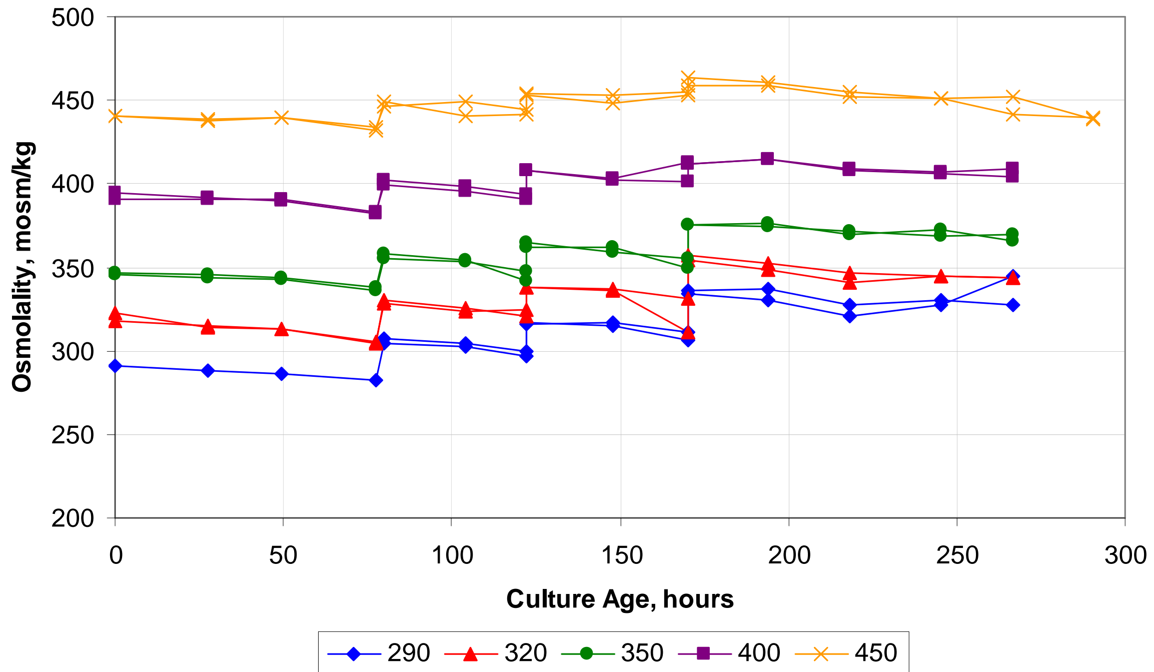
provide beneficial in the engineering of new industrial cell lines. Furthermore, osmotic shifts were found to be beneficial in increasing mAb concentrations. Increasing the yield of mAb during cell culture processes is one of the main goals of process development efforts in biopharmaceutical companies. Further optimization of the medium osmolality and timing of osmotic shifts would be an economical means of increasing mAb for industrial production.

The application of metabolomics to the cell culture field has a lot of potential for growth and future studies. The development of an easy and controlled quenching procedure to quickly cease cellular metabolism is a must to ensure true data collection when monitoring intracellular metabolites. In addition, improvements to separation techniques and metabolite databases, to accurately evaluate and identify the thousands of metabolites present in mammalian cells, is required.

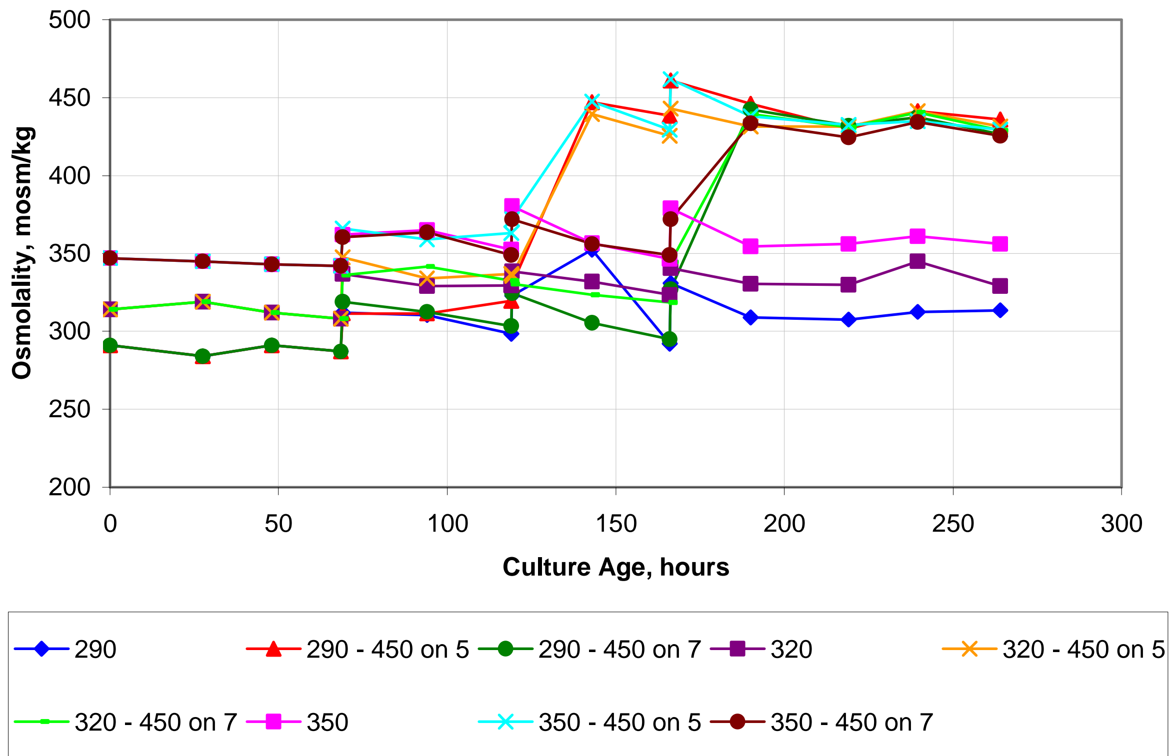
Appendix 1 Graphs of Osmolality Levels during Experiments



Appendix 1 Figure 1: Osmolality Levels during Batch Phase Experiment (Chapter 4).



Appendix 1 Figure 2: Osmolality Levels during Fed-Batch Experiment (Chapter 5).



Appendix 1 Figure 3: Osmolality Levels during Osmolality Shift Experiment (Chapter 6).

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