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Characterization of Chinese Hamster Ovary Cell Performance with Pentanoic Acid and N-Acetyl Cysteine Treatment

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CHARACTERIZATION OF CHINESE HAMSTER OVARY CELL PERFORMANCE

WITH PENTANOIC ACID AND N-ACETYL CYSTEINE TREATMENT

by

Joseph Camire

A thesis submitted in partial fulfillment
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of

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in

Biological Engineering

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ABSTRACT

Characterization of Chinese Hamster Ovary Cell Performance

with Pentanoic Acid and n-acetyl cysteine Treatment

by

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Increasing the volumetric titer of recombinant therapeutic proteins is a key objective in bioprocess manufacturing development. Carboxylic acids have demonstrated the ability to improve synthesis of recombinant proteins in industrially important cell lines such as Chinese Hamster Ovary (CHO) cells. The improvement in the production of recombinant proteins has been linked in a number of molecules to the inhibition of histone deacetylase, leading to increased transcription of genes. However, carboxylic acids have been shown to promote an apoptotic response in CHO cell culture. Pentanoic acid is a carboxylic acid that has demonstrated lower apoptotic response in culture relative to the carboxylic acid butanoic acid, while maintaining the ability to improve recombinant protein production.

Supplementation of cultures with anti-oxidants has shown the ability to reduce the apoptotic response of butanoic acid supplementation leading to increased therapeutic protein production. Here we show improved production with pentanoic acid and further

improvements combining induction with supplementation of the antioxidant n-acetyl cysteine (NAC).

Pentanoic acid induction of CHO cell cultures was studied in time-course experiments looking at growth, productivity, and apoptosis. Pentanoic acid was shown to reduce the number of cells entering early apoptosis relative to butanoic acid by 15.4%. The supplementation of butanoic acid and pentanoic acid treated cultures with n-acetyl cysteine reduced the population of cells entering early apoptosis by 5.3% and 10.0%, respectively. A 19.5% increase in productivity was observed in pentanoic acid treated cultures when supplemented with n-acetyl cysteine. This research provides evidence for a culture supplementation method that can be used in the optimization of biopharmaceutical manufacturing processes.

(79 pages)

PUBLIC ABSTRACT

Characterization of Chinese Hamster Ovary Cell Performance

with Pentanoic Acid and n-acetyl cysteine Treatment

Joseph Camire

To improve the cost of producing protein therapeutics manufacturers can attempt to increase the amount of protein produced per unit volume. Some small fatty-acids have shown the ability to increase protein concentrations in some manufacturing systems. An example system is in the industrially important cell line derived from a Chinese Hamster Ovary (CHO). A drawback of adding these fatty-acids is they can increase the rate of cell death.

A potential way to minimize the cell death rate, caused by adding fatty-acids, is to also add additional anti-oxidants. Through a series of studies, we demonstrate production with different fatty-acids, butanoic acid and pentanoic acid, with the addition of the antioxidant, n-Acetyl cysteine (NAC).

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I give special thanks to my family for their patience as I have worked through the process.

Joseph Camire

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1   Background and Significance

Biological pharmaceuticals play a significant role in human health care ranging

from cancer therapy to the breakdown of blood clots during a heart attack. These

biopharmaceuticals drugs are principally produced by recombinant protein technologies,

where gene sequences foreign to a biological host, are used to produce a non-native

protein, in the host cell system. The demand for biopharmaceuticals is large and is

expected to continue to grow over the coming decades. A large number of

biopharmaceuticals rely on mammalian cells as a production vehicle with their ability to

produce bioactive post-translationally modified glycoproteins.

Manufacturing process optimization with a target of increasing the volumetric

production of recombinant proteins can reduce cost and time of the manufacturing

process. A number of carboxylic acids have demonstrated the ability to improve

synthesis of recombinant protein in industrially important cell lines such as Chinese

Hamster Ovary (CHO) cells.   The increased productivity attributed to carboxylic acid

has been attributed to the hyper-acylation of histones. Histones are proteins that

deoxyribonucleic acid (DNA) winds around to create chromatin structures in eukaryotic

cell nuclei. The hyperacetylation of the histone unwinds the DNA leaving it more readily

available for transcription, leading to increased gene translation and productivity.

Butanoic acid is a carboxylic acid that has been shown to block cell proliferation in the

G1 cell cycle phase, this may also be a source of increased productivity in some cell lines

However, butanoic acid supplementation of cultures has been shown to promote an apoptotic response in CHO cell culture. An alternative carboxylic acid, pentanoic acid, has been shown to enhance the volumetric productivity of recombinant proteins (Liu et al. 2001).

Supplementation of cultures of cells with anti-oxidants has shown the ability to reduce the apoptotic response to carboxylic acid supplementation leading to increased therapeutic protein production. Antioxidant addition to cultures can improve post-treatment performance of cultures by minimizing apoptosis.

With an optimization target of increasing recombinant protein production in CHO cell lines a screen of multiple carboxylic acids was performed. Selected carboxylic acids were then screened with an antioxidant for further improvement of recombinant protein production.

Here we show improved production with pentanoic acid and further improvements combining induction with supplementation of the antioxidant n-acetyl cysteine (NAC).

1.2   Hypothesis

Small molecules can enhance the level of heterologous recombinant protein production. However, it has been observed that treatment with small molecule enhancers can induce apoptosis in the cultures. Antioxidants addition to these cultures can improve post-treatment performance of cultures by minimizing apoptosis.

1.3   Research Objectives

As a screening tool, analyze the performance of components as single parameters to observe does response concentration curves. Using a scale-down model 96 well microtiter plate culture system, gradients of test components will be analyzed over initial ranges of 0-20mM. The objective is to determine response concentrations of test components.

Design and analyze experiments looking at primary and secondary effects of combinations of selected small molecules and antioxidants. Assuming that Small Molecule Enhancers improve volumetric productivity, but have a negative impact on growth kinetics and that antioxidants show neutral to improved productivity or growth kinetics a factorial design of experiment is proposed to explore the possibility of improved performance from antioxidant addition to Small Molecule Enhancer induced cultures.

Explore the cause of reduced culture performance following induction with Small Molecule Enhancers and any positive effects from treatment with antioxidants. Apoptosis stimulation will be explored as an explanation for reduced culture growth performance after addition of Small Molecule Enhancers. Assuming apoptosis explains reduced culture performance, the effect of addition of anti-oxidants on apoptosis will be explored.

1.4   References

Liu CH, Chu IM, Hwang SM. 2001. Pentanoic acid, a novel protein synthesis stimulant for Chinese hamster ovary (CHO) cells. Journal of Bioscience and Bioengineering 91(1):71-75.

CHAPTER 2

LITERATURE REVIEW

2.1   Biopharmaceuticals

Over the last 35 years biopharmaceuticals have played an increasingly important

role in human and animal health.   As opposed to traditional chemical pharmaceuticals,

typified by small molecule drugs, biopharmaceuticals are biotechnology-derived.

Biopharmaceuticals encompass a broad range of treatments for areas that include

arthritis, enzyme replacement, allergy, cancer therapy, infertility, cardiology,

inflammatory diseases, blood disease, myocardial infarction, cystic fibrosis, organ

transplant, and tissue growth and repair. Within the broad category of

biopharmaceuticals are a variety of compounds and treatments; nucleic acids including

gene therapies, vaccines, cell therapy, and therapeutic proteins. Within therapeutic

proteins are three main categories; recombinant proteins, monoclonal antibodies, and

purified proteins. An example of a recombinant protein is Octocog (Table 1), a blood

enzyme known as Factor VIII. It is used as an enzyme replacement therapy in the

treatment of hemophilia A. An example of a monoclonal antibodies is Adalimumab

(Table 1) a recombinant human IgG1 that targets human tumor necrosis factor-alpha

(TNF-alpha). It is a glycosylated protein that contains 1,330 amino acids and has a

molecular weight of about 148 kilodaltons (Vena and Cassano 2007). Adalimumab is

able to bind TNF-alpha, a naturally occurring cytokine involved in inflammatory and

immune response (Arora 2007), thus blocking its ability to bind to the TNF-alpha cell

surface receptors, p55 and p75.

Table 1 Select list of FDA approved biopharmaceuticals that have reached blockbuster status (>$1 billion) in sales.(Bioplan Associates 2013)

Name	Product	Class	Cell Line
Adalimumab	TNF MAb	Monoclonal Antibody	CHO
Bevacizumab	VEGF MAb	Monoclonal Antibody	CHO
Epoetin alfa	EPO	Recombinant Protein	CHO
Etanercept	TNF receptor-IgG Fc	Monoclonal Antibody Fragment	CHO
Infliximab	TNF MAb	Monoclonal Antibody	SP2/0
Insulin Glargine	Insulin Glargine	Recombinant Protein	Escherichia coli
Octocog	Factor VIII	Recombinant Protein	CHO
Pegfilgrastim	G-CSF Pegylated	Recombinant Protein	Escherichia coli
Ranibizumab	VEGF MAb Fab	Monoclonal Antibody Fragment	Escherichia coli
Rituximab	CD20 MAb	Monoclonal Antibody	CHO
Trastuzumab	HER2 Receptor MAb	Monoclonal Antibody	CHO

In 2013 it was estimated that there were 435 biopharmaceuticals approved for use in the United States and Europe (Bioplan Associates 2013). Of the approximately 10,000 therapeutic in R&D, it is estimated that 40% are biopharmaceuticals. In 2013 it was estimated that biopharmaceuticals had a market size of about $165 billion and was growing at a 15% compounding annual growth rate. This market size represents approximately 15% of the estimated $1 trillion total pharmaceutical market. It is estimated that the biopharmaceutical market will grow to greater than $210 billion by 2015 (Technavio 2013). The biopharmaceutical industry group, PhRMA, estimated that in 2012 there was $48.5 billion spent on research and product development innovations (PhRMA 2013).   It is projected that this funding trend will continue with the potential for nearly 50% of pharmaceutical R&D projected to be devoted to biopharmaceuticals (Bioplan Associates 2013).

2.2   Monoclonal Antibodies

Within biopharmaceuticals, monoclonal antibodies are an important segment. Seven of the top ten selling biopharmaceuticals in 2011 were monoclonal antibodies or fragments of monoclonal antibodies. Monoclonal antibodies are natural occurring glycosylated proteins that play a major role in the body's immune system. They are monospecific to an antigen and are produced by B lymphocytes, a class of immune cells, in response to an antigen. Specific B lymphocyte only produces one specific antibody and have limited, to no proliferation capacity. In 1975, George Kohler and Cesar Milstein demonstrated that a specific B cell could be isolated and fused with a cancerous myeloma cell line. The B cell bringing the ability to produce a specific antibody and the myeloma cell conferring the ability to replicate rapidly and indefinitely. This process, known as hybridoma technology, ushered in a process to allow expanded production of a monoclonal antibody to a specific target molecule. One method for producing the B cell needed for the fusion is to expose a mouse to an antigen in two phases, a priming dose and a booster dose. Following treatment with the antigen a sample of the spleen can be harvested. Following fusion with the myeloma cell line the hybridoma population can be screened for a population producing the antibody of interest.

Immune recognition and response in humans to IgG produced in other species prompted the move to more human-like IgG. Most therapeutics antibodies are either humanized or are fully human IgG and are produced as recombinant glycoproteins in mammalian cells (Beck et al. 2008). Humanized monoclonal antibodies are genetic constructs that contain both human and non-human gene sequences for specific antibody

regions, the conserved genetic regions coming from human sequences and the variable genetic sequences, specific to an antigen, regions coming from species donating the B-cell Lymphocyte.

MAb are generally expensive therapies that can cost more than $100,000 per year or over the course of full treatment (Bioplan Associates 2013). The high cost of MAb treatment can be attributed to low potency and short half-life, requiring large and frequent dosages. These factors lead to large amounts of MAb being needed to treat patient populations. It is estimated that around 90% of the world-wide production capacity for mammalian cell culture is for MAb production (Bioplan Associates 2013). Much of this production requires the use of large volume bioreactors at the 10,000 L scale.

2.3   Recombinant DNA Technology

With the introduction of recombinant protein production in 1974 by Herbert Boyer at University of California San Francisco and Stanley Cohen at Stanford it was possible to engineer systems for the manufacturing of biopharmaceuticals, rather than purification of proteins from source tissue and organs. Recombinant proteins and glycoproteins are the products of exogenous gene sequences. These recombinant genes can be spliced into the genome of alternative cells to be used as a production system. The alternative cell line systems provide the gene replication, transcription, translation, and if required the secretion of the targeted proteins and glycoproteins. Recombinant gene expression cassettes typically contain promoters, initiation sequences and may contain introns and exons, and coding sequences such as polyadenylation signals.

The predominant production system for biopharmaceuticals use recombinant DNA technology. Early recombinant engineered systems relied on proteins produced in bacterial systems. E. coli are an example of a bacterial production system used to produce recombinant proteins that lack typical mammalian post-translational modifications. When complex, human-like post-translational modifications are needed then mammalian cells are the typical system used for biopharmaceutical production. The primary post-translational modification is protein glycosylation, a modification where polymers of sugars are attached to proteins.

These drugs have played an important role in human health care over the last 30 years (Brown 2005; Carter 2011).   A typical development process for production using recombinant DNA would start with identification of a target gene sequence (Figure 1). An expression cassette may be developed then used in a transient expression system. Transient expression systems rely on introduction of the recombinant DNA into the cell line, but the gene isn't incorporated in the cell genome. However, using the native cellular processes the gene of interested is transcribed and then the target proteins in translated from the transcribed mRNA. The resulting recombinant protein can then be tested for bioactivity and toxicity. Transient production systems are typically low-level production systems, thus requiring the production of stable cell line for optimized protein expression. Selected genes are then used to produce stable cell lines that have the gene incorporated into the production cell line genome.

Within biopharmaceutical production, Chinese Hamster Ovary cells are one of the predominate cell systems used, because of relatively straightforward genetic

manipulation of the cell line, growth of the cell line is rapid and can be optimized, and general acceptance of use of this cell line by government regulatory bodies such as the FDA and EMEA.
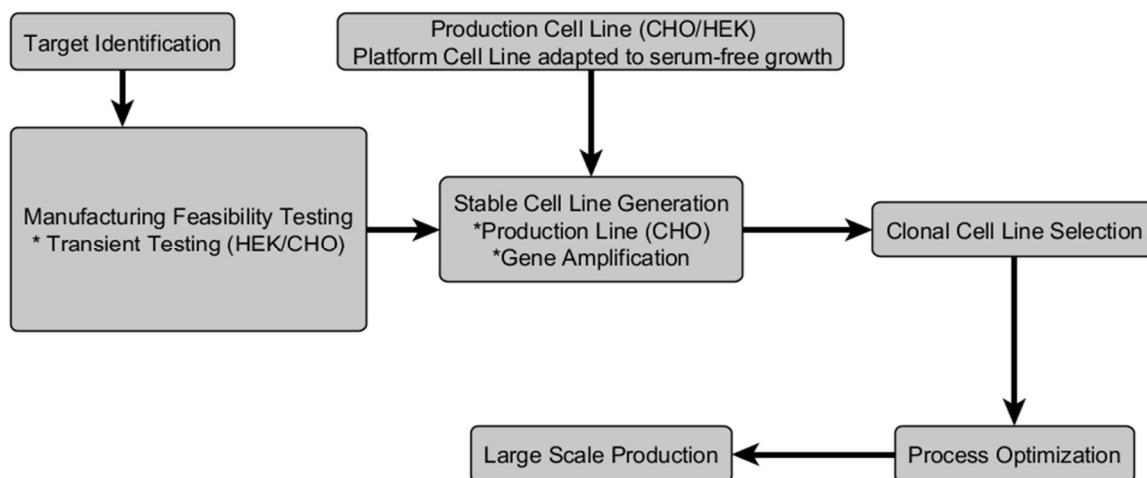
Figure 1 **Flow diagram of development of a recombinant production cell line.**

Recombinant cell lines used for production are generally screened in a clonal selection process, to eliminate non-producers and select clones with higher production rates. Chosen clones can then be optimized in scale-down model systems of full large-scale production processes.

2.4   Production of Biopharmaceuticals.

The production of human therapeutics that require unique processing to be active, relies on mammalian cells because of their ability to correctly fold, perform post-translational modifications, and secrete proteins that aren't possible in microbial and plant-based systems. (Omasa et al. 2010a)

Mammalian cell culture can be traced back to organ culturing techniques in the 19th century. By the 1950's many procedures had been developed for culturing cells outside of mammalian cells. Of main importance in advancing the process, at this time, were studies in the production of virus. To facilitate growth in vitro culture conditions were created similar to the environment in vivo, with serum being a primary constituent in most culturing systems. Serum being an animal-blood product provided many important factors, including, metabolites, growth factors, mitogens, lipids, and proteins. To minimize the use of serum, a costly and variable component, basal liquid formulations consisting of carbohydrates, to supply energy, amino acids, vitamins and salts were developed. Although an undefined supplement, serum played an important role in culturing, supplying growth factors, lipid carriers, antioxidants, protease inhibitors, however, its undefined character could lead to variability in performance. Its collection from abattoirs as a byproduct of the beef industry made it subject to variability in supply with the result that as a commodity it price could fluctuate dramatically. The development of basal media formulations allowed for serum to be reduced to levels of 5-15% of culture volume and in some cases to be eliminated altogether. When biopharmaceutical production started to develop and grow in the 1970's and 1980's, serum was still an important culture supplement for production. By the 1990's formulations and culturing methods were beginning to be introduced for protein production without the use of serum. Because of product life cycles, cost to develop new processes and systems, there are many legacy products that are still manufactured using serum.

Along with advancements in culture media formulations, systems for manufacturing have advanced over the last three decades. Processes for biopharmaceutical production include batch, fed-batch, and perfusion or variations of these systems. Some manufacturing processes rely on batch methods for their simplicity, although most processes are currently fed-batch processes. This manufacturing method lends itself well to serum-free formulation advancements in the last two decades, since known depleted components could be fed into the process during culture processes. Although perfusion-based systems of production have been developed, views that they are complex have limited their use. However, current pushes in development are focusing on perfusion as ideal manufacturing system of future production platforms.

After identification of the product of interest and production cell line generation, the next step is production of a Master Cell Bank. A production process (Figure 2) would then start with a Working Cell Bank being produced. Typically, cell banks consist of small volume aliquots of cells preserved in cryostorage, in volumes in the 0.5 to 10mL range.
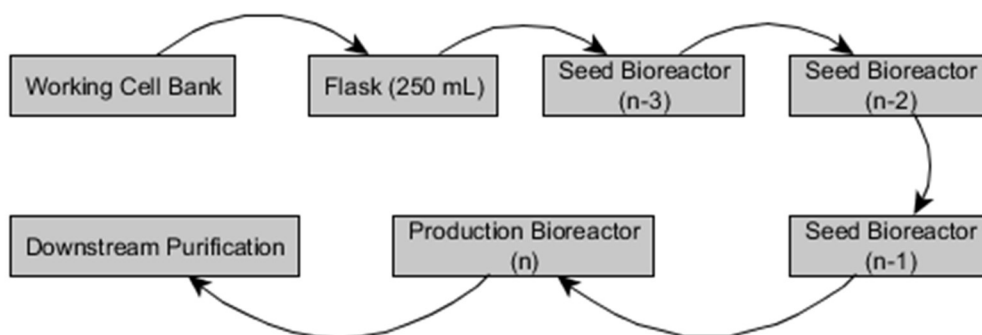
Figure 2 Bioprocess Schematic showing seed train procession.

Production of biopharmaceuticals in mammalian cell lines remains an expensive and complex process. Increasing the volumetric titer of recombinant therapeutic proteins is a key objective in bioprocess manufacturing development. To achieve higher product titers, driving down cost, researchers have employed multiple strategies. A typical process will use suspension cells (Figure 2), were cells grow detached from contact surfaces. Processes over the last 20 years have increasingly been engineered to use serum-free cultures to minimize cost in the production bioprocess and in purification of target proteins in downstream processes.

Figure 3 Photomicrograph of suspension CHO Cells settled on to tissue culture flask bottom.

Modifications in the culture conditions and genetic based engineering of cell lines used for production of recombinant targets have played a significant role in optimization of production. A frequent optimization target is arresting the cells in G1 cell cycle phase, due to reduced cell growth and higher protein production. Three methods capable of the

growth arrest are: temperature shift, cell engineering, and bioactive chemicals.

Temperature shifting resulting in cell cycle arrest can improve transcription, translation,

cytoskeletal rearrangement, with reduced metabolic activity. The reduced metabolism in

the cells is reflected in reduction in glucose metabolism and oxygen consumption (Kumar

et al. 2007; Meleady 2007). Cell engineering approaches can target the integration of

genes for over expression such as Bcl-2, a regulatory protein involved in apoptosis

mediated cell death (Tey et al. 2000).
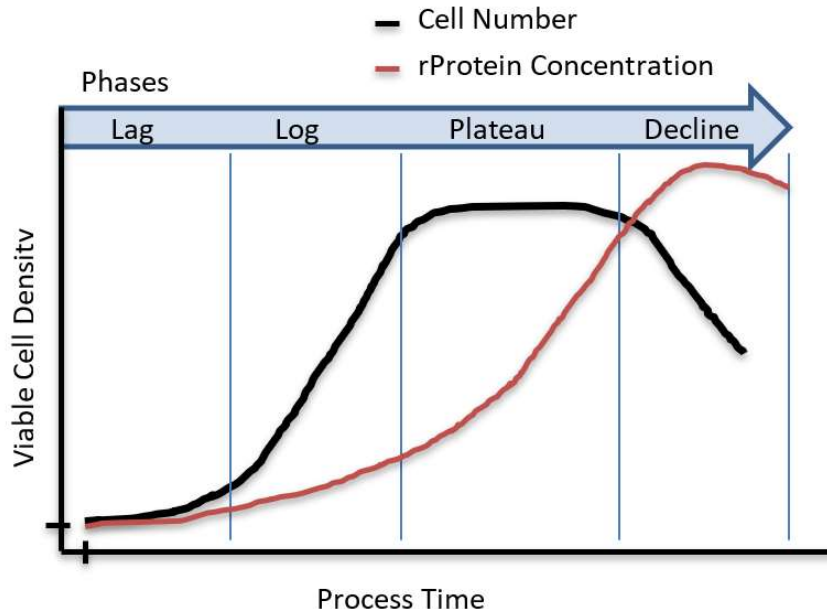
Figure 4 Schematic of typical batch/fed-batch profile of culture kinetics.
Production of target protein frequently shows peak accumulation in plateau and the
decline phase of culture.

2.5   Post Translational Modifications

It has been found that many protein based biologics require post translational

processing for biological activity (Butler 2005). Glycans on the surface of proteins play

an important role in immune function. They are generally specific to a species and non-

native glycan structures can trigger immune response and high clearance rates of foreign proteins from the body. Improperly glycosylated proteins can trigger immunogenic and allergic response and accelerate clearance of the protein from the body. Glycosylation can be affected by the choice of manufacturing process and clonal variation.
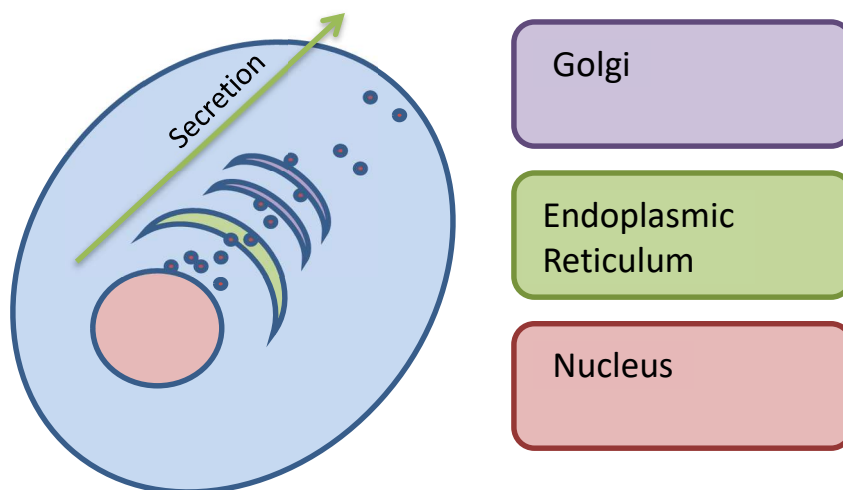
Figure 5 Mammalian Cell rProtein processing
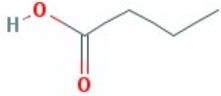
(Note: Similar to NEB diagram (Omasa et al. 2010b))

2.6   Small Molecule Enhancers
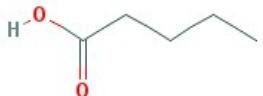
Bioactive molecules can target many cellular mechanisms ranging from metabolic to genetic changes. Important to bioprocessing are molecules that can stimulate the improved production of recombinant proteins. A wide variety of molecules have been studied for this purpose, for example quinoline thioethers (Kazi et al. 2010), dimethyl sulfoxide (Fiore et al. 2002; Li et al. 2006; Liu and Chen 2007; Liu et al. 2001b; Wang et al. 2007), and aurintricarboxylic acid (Liu et al. 2001a). Additionally, broad classes of small molecule in the categories of aromatic carboxylic acids, hydroxamic acids, and acetamides have been studies (Allen et al. 2008).

Short chain fatty acid (SCFA), a class of saturated monocarboxylic acids, have been shown to have many biological functions, including regulating gene transcription (Meng et al. 1999) and inhibition of histone deacetylase (Augenlicht et al. 2002). There are a number of SCFA that are important in cells, varying in their carbon backbone length; acetate (C2), propionate (C3), butanoate (C4), pentanoate (C5), and caproate (C6). The most widely studied of these, for increased protein production in bioprocessing, has been butanoic acid (C4) and has demonstrated a wide effect on cells. Butanoic acid has been implicated in cell proliferation, gene transcription regulation, differentiation, and apoptosis (Augenlicht et al. 2002; Kim and Lee 2002; Sung et al. 2004). Butanoic acid has been shown to stimulate the gene coding for metallothionein. Metallothionein (MT) are small cysteine-rich metal binding proteins. MT plays an important role in maintaining zinc and copper homeostasis in cells and can detoxify harmful metals such as cadmium (Liu et al. 1992).

Table 2 Select table of SCFA and the antioxidant n-Acetyl Cysteine (Chemical Structure Source PubChem Compound http://pubchem.ncbi.nlm.nih.gov/)

IUPAC Name:	Common Name	CID[1]#	MW	Molecular Formula	2D Structure
Butanoic acid	Butanoic Acid	264	88.11	$C_4H_8O_2$	

[1] http://pubchem.ncbi.nlm.nih.gov/citations.html

Pentanoic acid	Valeric Acid	7991	102.13	$C_5H_{10}O_2$	

Butanoic acid was demonstrated to increase protein production by Gorman et al (Gorman et al. 1983). An increase in the enhancer-dependent transcription of an SV40 promoter was approximately 30-fold higher in Hela Cells. The increased production of chloramphenicol acetyltransferase (CAT) in these cells was attributed to the hyperacetylation of histones, thus opening the chromatin structure in critical regions spanning the enhancer and the adjacent promoter, leading to increased transcriptional efficiency.

The inhibition of histone deacetylase is one of the most widely studied effects of butanoic acid treatment. Inhibition of histone deacetylase leads to hyper-acetylation of histone molecules, a class of DNA packaging molecules, which opens gene regions making them more readily available for transcription, leading to increased mRNA levels, with increasing translation and productivity. However, butanoic acid has a well-documented effect of inducing apoptosis in cells it is used to treat.

Additionally, Butanoic acid has been shown to block cell proliferation in the G1 cell cycle phase, this may also be a source of increased productivity in some cell lines. A limitation to the use of carboxylic acids is increased levels of apoptosis observed in treated cultures.

The effect of butanoic acid induction of the production of recombinant human thrombopoietin (hTPO) produced in Chinese Hamster Ovary cells has been studied for its

impact on the heterogeneity and biological activity of the protein (Sung et al. 2004). The human thrombopoietin protein is a highly glycosylated protein with six N-glycosylation sites and 24 O-glycosylation sites. The post-translational glycosylation of recombinant proteins has been shown to be effected by the treatment of cultures of CHO cells with sodium butyrate (Chung et al. 2001; Santell et al. 1999). Sung et al. were able to achieve a peak hTPO concentration of 82.2 ± 5.6 ug/mL treating cells growing in exponential phase with 3 mM butanoic acid. This group demonstrated that compared to a control culture, a 6.4-fold increase in specific productivity, q_{TPO}, and a 3.3-fold increase in volumetric productivity on day 7 of the culture. The butanoic treated culture demonstrated diminished protein quality in the form of a lower level of acidic forms of hTPO being produced. Additionally, they demonstrated lower biological activity of the butanoic acid treated hTPO.

Oster et al. demonstrated improved productivity for the recombinant production of the plasma extracellular membrane-anchored enzyme, γ-glutamyl transferase (GGT, EC2.3.2.2) by sodium butanoate (Oster et al. 1993). Using a cytomegalovirus immediate early promoter in a CHO cell line, they demonstrate a 3 to 5-fold increase in production of GGT. They observed increase levels of rGGT mRNA production following butanoic acid induction. The resulting rGGT was characterized and demonstrated to be equivalent to non-induced control culture rGGT.

The use of short-chain carboxylic acids such as pentanoic acid (Liu et al. 2001c) has been shown to enhance the volumetric productivity of recombinant proteins produced

in bioprocesses. The addition of antioxidants in cultures treated with the carboxylic acid, butanoic acid, has lowered the levels of apoptosis observed (Oh et al. 2005).

Carboxylic acids have demonstrated the ability to improve synthesis of recombinant protein in industrially important cell lines such as Chinese Hamster Ovary (CHO) cells. However, some carboxylic acids have been shown to promote an apoptotic response in CHO cell culture. Chi-Hsien Liu, et al(Liu et al. 2001c), researched the small molecule enhancement of protein production by a group of nine carboxylic acids by themselves and in combination. They found that the five-carbon carboxylic acid, pentanoic acid, showed higher relative protein titer increase compared to the well publish butanoic acid counterpart. They also demonstrated results for lower apoptotic cell effect. A number of carboxylic acids, formate, acetate and 2-methyl butyrate showed slight enhancement effects on cell growth and production. Some carboxylic acids, such as L-alpha-amino-n-butanoic acid showed slight enhancements in growth but no enhancement of production. It was demonstrated that when the carbon backbone range was between 3 to 5 carbons, there was a marked increase in titer enhancement. Slightly higher growth and a better protein production enhancement were observed for the 5 carbon pentanoic acid over the 4 carbon butanoic acid. To try and explain these results the authors looked at apoptosis as an explanation for the results. Butanoic acid is a well-known enhancer of protein production (Lamotte et al. 1999; Mimura et al. 2001), but also has a well-documented apoptotic affect (Kim et al. 2003). In these studies, it was found that butanoic acid had about double the apoptotic affect that pentanoic acid had using an arbitrary apoptosis ratio. The method used was a cell death detection ELISA kit looking

at DNA fragmentation. Of note in these studies is the relative low producer used in the studies. Whereas a common fusion antibody produces from 500mg to 5 g per liter, the cell line used by Liu only produced a maximum of 620 ug per liter, or about 1:800 of a common producer cell line.(Preithner et al. 2006)

It is important that a good process strategy be used to maximize productivity of culture(Franco-Lara and Weuster-Botz 2005). A number of newer small molecule enhancers have been developed that are based on aromatic hydrocarbons. These haven't been compared to pentanoic acid (Allen et al. 2008).

2.7   Apoptosis

Apoptosis is programmed cell death triggering a biochemical cascade of events leading to cell destruction. The cascade of the events includes cell blebbing, fragmentation of the DNA and the nucleus.
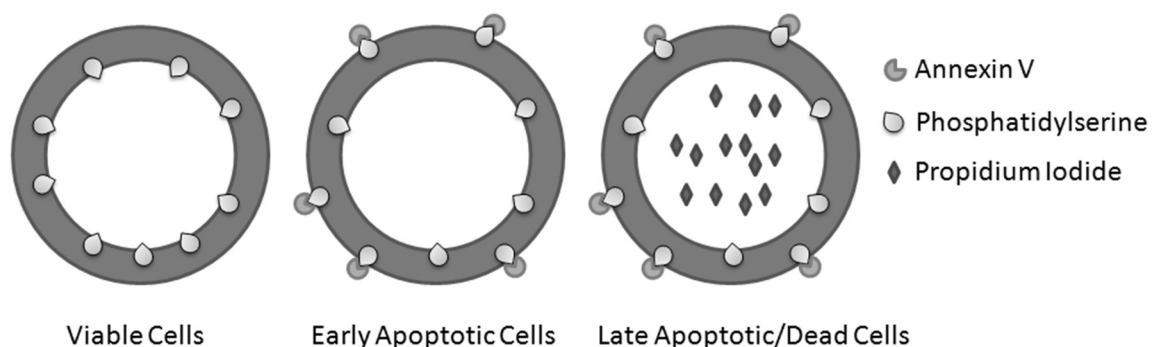
Figure 6 Diagram of apoptosis process in cells with illustration of Annexin V and Propidium Iodide analysis of cell state.

2.8   Antioxidants
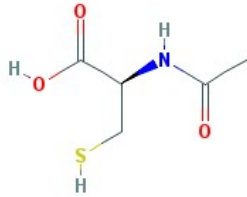
Oxidative stress is a major factor limiting production and quality of product produced in recombinant systems. Many antioxidants have been studied for their protective and enhancing effect on cells. Antioxidants demonstrate the ability to reduce stress in the endoplasmic reticulum, a key site of glycosylation (Malhotra et al. 2008).

2.8.1   Use of Antioxidants in combination with Small Molecule Enhancers

Antioxidants have been used extensively in cell culture. An example is the work done by Oh et al. (Oh et al. 2005). This group evaluated a panel of eleven antioxidants using a Plackett-Burman statistical design. In their work they demonstrated that of the panel tested only n-Acetyl cysteine (NAC), seen in Table 3, showed increased longevity of cell culture and increased production of the target protein, recombinant interferon-β-1A (IFN-β). In butanoic acid treated cells, NAC treatment was able to extend culture longevity greater than 8 days and increased recombinant INF-β production 2-fold when compared to control cultures that were treated only with butanoic acid. They further demonstrated that the glycosylation patterns of the INF-β, studied using isoelectric focusing (IEF), showed increased levels of lower pI isoforms compared to the control run. The recombinant INF-β from NAC treated cultures had a 17.7% higher level of sialic acid content when analyzed by HPLC.

Table 3 Chemical Description of n-Acetyl cysteine.

Common Name	IUPAC Name:	CID[2]#	MW	Molecular Formula	2D Structure
n-Acetyl Cysteine	(2R)-2-acetamido-3-sulfanylpropanoic acid	12035	163.19	$C_5H_9NO_3S$	

2.9   Chinese Hamster Ovary Cell Line

Production of biopharmaceuticals has come to rely on mammalian cells; in particularly they have come to rely on Chinese Hamster Ovary cells. The predominant cell line used in the production of biopharmaceuticals is the Chinese Hamster Ovary (CHO) cell line (Jayapal et al. 2007). This cell line was derived from a female *Cricetulus griseus* hamster in 1957, by Dr Theodore Puck in the Department of Medicine at the University of Colorado. *Cricetulus griseus* have a low chromosome number (2n=22). The key cell line used in biopharmaceutical production, derived from C. griseus, CHO-K1, has been shown to be heteroploidy. It was observed early on that the cell line was relatively easy to cultivate in vitro and had a good growth rate, with specific growth rates that can range from 0.04 - 0.06 hr^{-1}.

Important to the development of CHO cells for biopharmaceutical production were observations of the CHO genome being relatively easily manipulated and the development of expression and amplification systems. The dihydrofolate reductase

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[2] http://pubchem.ncbi.nlm.nih.gov/citations.html

(DHFR) selection and amplification system and the glutamine synthetase (GS) selection and amplification system (Camire 2000). The DHFR expression system relies on the critical path for DHFR in the production of nucleotides for DNA (Figure 7) synthesis and replication.
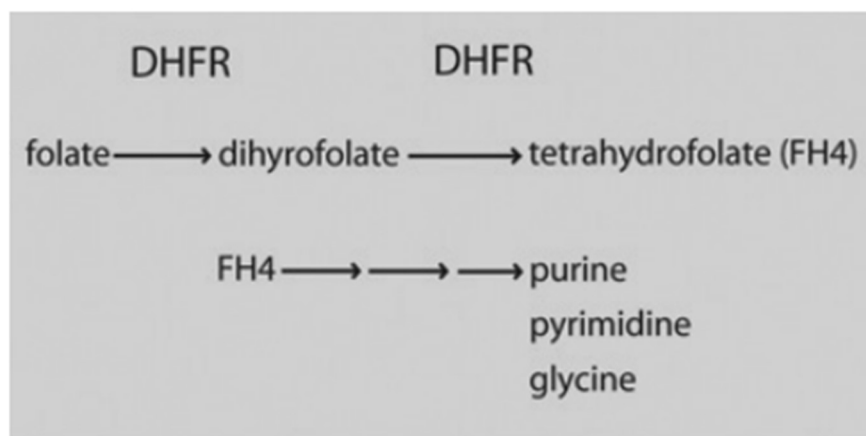
Figure 7 Schematic of DHFR enzyme process.

Important to the development of this expression system was the development of DHFR knockout cell lines in Dr. Larry Chasin's lab at Columbia University. The CHO DUKX B11 and the CHO DG44 cell lines are single and double allele knockouts of the DHFR gene, respectively, and were generated by the use of mutagens. In the absence of nucleic acids and the precursors hypoxanthine and thymidine, these cell lines cannot replicate and unsupplemented populations die off. Incorporating the DHFR gene into an expression cassette with a gene of interest allows the recombinant cell line to live in a selection medium without nucleic acid precursors and hypoxanthine and thymidine. Another important feature of this system is the dose response competitive inhibition of

the DHFR enzyme by the compound methotrexate (CAS Number 133073-73-1). By

increasing the concentration of methotrexate in a culture of CHO cells with an expression

cassette containing DHFR, successive populations of cells that have increasing copies of

DHFR and the corresponding gene of interest are selected for. In this way gene copy

number amplified populations can be selected that can have two to thousands of desired

genes (Kaufman et al. 1985).

The GS expression system relies on the conversion of glutamate and ammonium

to the amino acid glutamine (Figure 8). This system has been shown to be effective in

both knockout and wild-type cell lines containing a native GS gene. Similar to DHFR

expression systems the glutamine synthesis enzyme can be competitively inhibited by

methionine sulfoximine (CAS Number 15985-39-4).
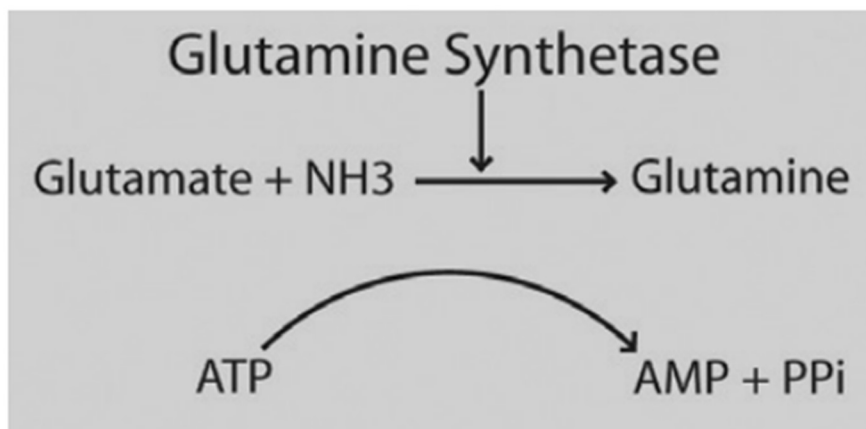
Figure

 8 Schematic of GS selection system.

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Chapter

SCREENING OF SHORT CHAIN FATTY ACIDS AND AN ANTIOXIDANT WITH

CHINESE HAMSTER OVARY CELLS

3.1   Introduction

Tissue plasminogen activator (tPA) is a 68 kDa monomeric serine protease (EC 3.4.21.68) produced primarily by endothelial cells. In blood, it is one of two major activators of plasminogen, the precursor of plasmin which is a serine protease that can dissolve fibrin blood clots. A recombinant form of the protein is produced as a biopharmaceutical drug, Activase®, for treatment of acute myocardial infarction, acute ischemic stroke, pulmonary thrombosis, and other blood clot conditions (Wagstaff et al. 1995). Its production is done in CHO cells and acts as a model for non-IgG biopharmaceutical recombinant protein production systems.

Bioactive chemicals can target many cellular mechanisms including protein expression. One of the targeted mechanisms is to induce cell stress to cause up regulation of protein production. A wide variety of molecules have been used to promote increased protein production in recombinant cell lines, for example quinoline thioethers (Kazi et al. 2010), dimethyl sulfoxide (Fiore et al. 2002; Li et al. 2006; Liu and Chen 2007; Liu et al. 2001b; Wang et al. 2007), and aurintricarboxylic acid (Liu et al. 2001a). Additionally, broad classes of small molecule in the categories of aromatic carboxylic acids, hydroxamic acids, and acetamides have been studies (Allen et al. 2008).

One of the most studied molecules for stimulation of protein production is butanoic acid. Butanoic acid is a short chain fatty acid (SCFA), a class of saturated monocarboxylic acids. SCFA have been shown to have many biological functions, including regulating gene
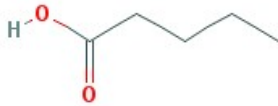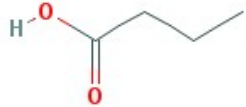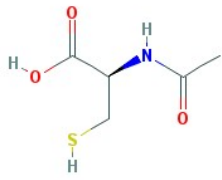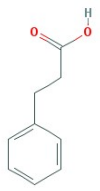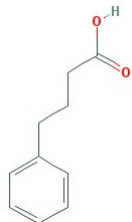
transcription (Meng et al. 1999) and inhibition of histone deacetylase (Augenlicht et al. 2002). There are a number of SCFA that are important in cells; acetate (C2), propionate (C3), butanoate (C4), pentanoate (C5), and caproate (C6). The most widely studied of these has been butanoic acid (C4) and has demonstrated a wide effect on cells (Table 3). Butanoic acid has been implicated in cell proliferation, gene transcription regulation, differentiation, and apoptosis (Augenlicht et al. 2002; Kim and Lee 2002; Sung et al. 2004). Butanoic acid has been shown to stimulate the gene coding for metallothionein. Metallothionein (MT) are small cysteine-rich metal binding proteins. MT plays an important role in maintaining zinc and copper homeostasis in cells and can detoxify harmful metals such as cadmium (Liu et al. 1992).

Butanoic acid was demonstrated to increase protein production of by Gorman et al (Gorman et al. 1983). An increase in the enhancer-dependent transcription of an SV40 promoter was approximately 30-fold higher in Hela Cells. The expression increases of chloramphenicol acetyltransferase (CAT) in these cells was attributed to the hyperacetylation of histones, thus opening the chromatin structure in critical regions spanning the enhancer and the adjacent promoter, leading to increased transcriptional efficiency.

The inhibition of histone deacetylase is one of the most widely studied effects of butanoic acid treatment. Inhibition of histone deacetylase leads to hyper-acetylation of histone molecules, a class of DNA packaging molecules, which opens gene regions making them more readily available for transcription, leading to increased mRNA levels increasing translation and productivity. However, butanoic acid has a well-documented effect of inducing apoptosis in cells it is used to treat.

Table 4 Small Molecules used in this screening study.

Compound/ Stock Concentration	CAS Number	MW	Abbreviation	Structure[3]
Pentanoic Acid 500 mM	109-52-4	102.1	PA	
Butanoic Acid 250 mM	107-92-6	88.11	BA	
N-Acetyl Cysteine 250 mM	616-91-1	163.19	NAC	
Hydrocinnamic 250 mM	501-52-0	150.17	HCA	
4-Phenylbutanoic Acid 250 mM	217-341-8	164.20	4PBA	

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[3] http://pubchem.ncbi.nlm.nih.gov/citations.html

3.2   Materials and Methods

3.2.1   Cell Line and Media

A Chinese Hamster Ovary cell line, CHO 1-15$_{500}$ (ATCC® CRL-9606™), producing human tissue Plasminogen Activator (tPA) was used for these studies. The cell line had previously undergone dihydrofolate selection and amplification with 50 nM methotrexate. The cell line was cultured using the chemically-defined serum-free medium CDM4CHO® (HyClone, Thermo Fisher Scientific, Waltham, Massachusetts) with 4mM L-Glutamine. Cell stocks were maintained in 100 mL cultures in 250 mL shake flask in a 37°C, 5%CO$_2$ incubator, passaged every 3 to 4 days.

3.2.2   Component Preparation

All compound tested were prepared in deionized process water and were sterile filtered using 0.2 um sterilizing grade bottle top filters. Butanoic Acid, n-Acetyl –cysteine, hydrocinnamic acid, 4-Phenylbutanoic acid were prepared at a concentration of 250mM and Pentanoic acid was prepared at a concentration of 500mM.

3.2.3   Bioprocess Experiments

Disposable 125mL Erlenmeyer shake flasks were used for scale-down culture analysis of the short chain fatty acids and n-acetyl cysteine. Experimental conditions were cultured using a base formulation of CDM4CHO® (HyClone, Thermo Fisher Scientific, Waltham, Massachusetts) in a fed batch process using a custom formulation process supplement PS320 (HyClone, Thermo Fisher Scientific, Waltham, Massachusetts). The PS320 process supplement contains carbohydrates, amino acids, and vitamins. This formulation, PS320:60$_{8.0}$, was prepared at a concentration of 60g/L by using adjustments to pH 9.0 with 1N NaOH followed by

adjustment with 1.0 HCl to pH 8.0 followed by the addition of 3.2 g/L sodium bicarbonate. The formulation was then sterile filtered using a 0.2 um sterilizing grade bottle top filter.

A single 2.8 L disposable Fernbach flask was seeded at an initial volume of 600 mL ($0.8V_f$) at a cell density of 250,000 cells/mL on day 0. The culture was fed two times with 75 mL ($0.1V_f$) PS320:60$_{8.0}$ on day 3 and on day 5. Following the feeding of the culture on day 5, cells were distributed into independent culture flasks in preparation for treatment with designated culture test conditions. Cultures were sampled on days 0, 3, 5, 7, and 10 for cell quantitation, viability analysis and product titer.

3.2.4   Cell Quantitation

Cell density and viability were determined using Trypan Blue dye exclusion assays. Microtiter plate cultures were counted using on a Cellavista® Cell Imager (SynenTec, Elmshorn, Germany). Shaker flask cultures were counted using an automated ViCell® XR Cell Viability Analyzer (Beckman Coulter, Fullerton, California). Aliquots for cell counting were counted immediately after sampling.

3.2.5   Product Titer Analysis by Enzyme-Linked Immunosorbent Assay

Product titer was determined using a sandwich enzyme-linked immunosorbent assay (ELISA). An affinity purified sheep anti-tPA IgG was used as a capture antibody by coating a standard ELISA 96 well plate. A reference standard curve was generated using a concentrated and titered recombinant tPA control in the range of 3.16 to 1000 ug/mL. Samples from culture conditions which had previously been stored frozen at -20°C were thawed and dilutions were prepared in phosphate buffered saline (PBS) at a dilution factor of 1:2000. Samples were then applied to the capture antibody prepared ELISA plates. A sheep anti-tPA conjugated to horse-

radish peroxidase was used as the detecting antibody. A stable liquid one-step 3,3′,5,5′-

tetramethylbenzidine (TMB) liquid was used as a substrate for colorimetric analysis. A

SpectraMax M2 (Molecular Devices, Sunnyvale, CA) was used to analyze the assay plates at a

detection wavelength of 450 nm.

$$IVCD = \int_{t_0}^{t_f} x \, dt$$

$$IVCD = \sum_i \left[\left(\frac{y_i + y_{i-1}}{2} \right) * (x_i - x_{i-1}) \right]$$

3.3   Results and Discussion

To determine the effect of multiple small molecules and an antioxidant for their potential

to enhance volumetric protein production, a CHO cell line producing tPA was subjected to

gradients of the components (Figure 9).
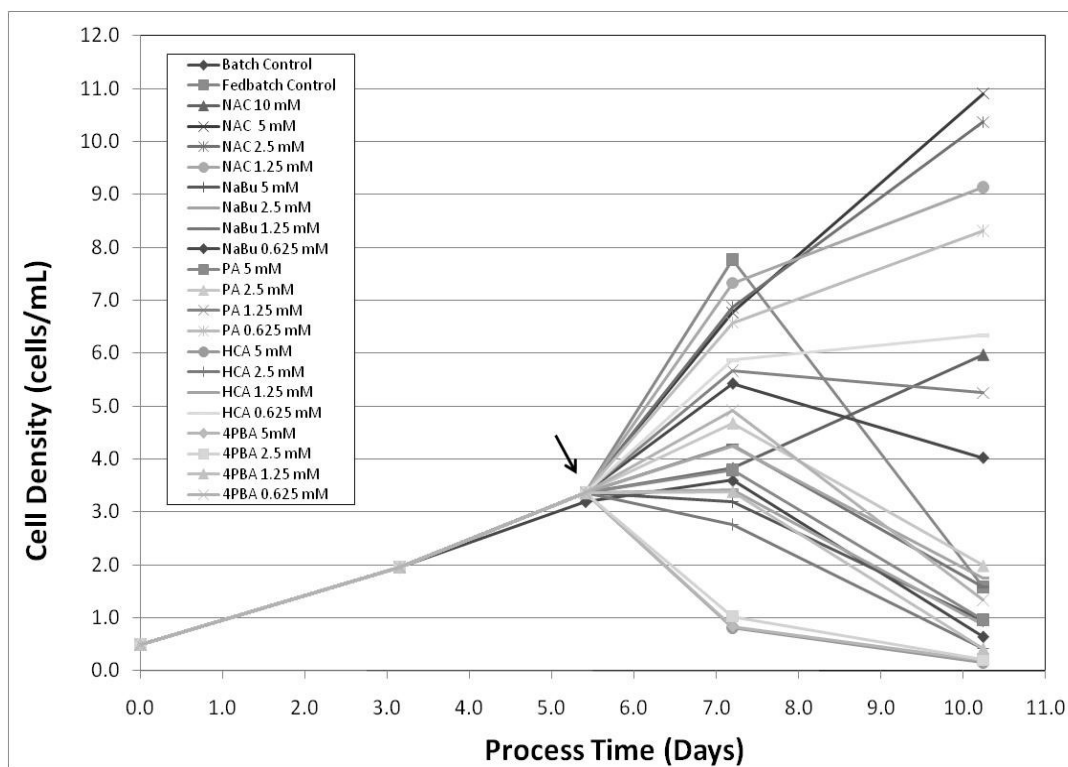
Figure

 9 Growth promotion analysis of carboxylic acid treatment conditions.

The fed batch control condition showed a large decline following day 7. A significant improvement was observed in the cell growth for samples treated with n-Acetyl-Cysteine. The n-Acetyl-Cysteine cell conditions didn't suffer the precipitous decline in cell density and viability observed with the fed-batch control. This may be attributed to a large decline in viability at this point (Figure 10).
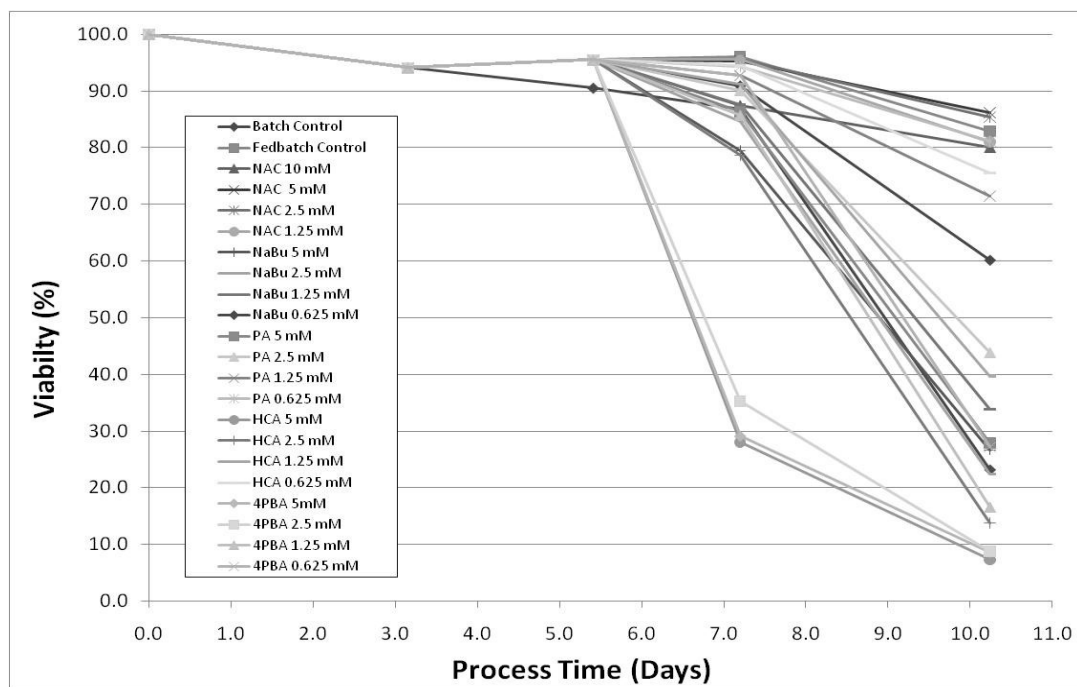
Figure

10 Plot of viability for carboxylic acid treatment conditions.

Higher concentration treatments at the 5 mM level of Sodium Butyrate, Pentanoic acid, Hydrocinnamic acid, and 4-Phenylbutyric acid showed almost complete inhibition of cell growth starting at time of treatment (Figure 11).
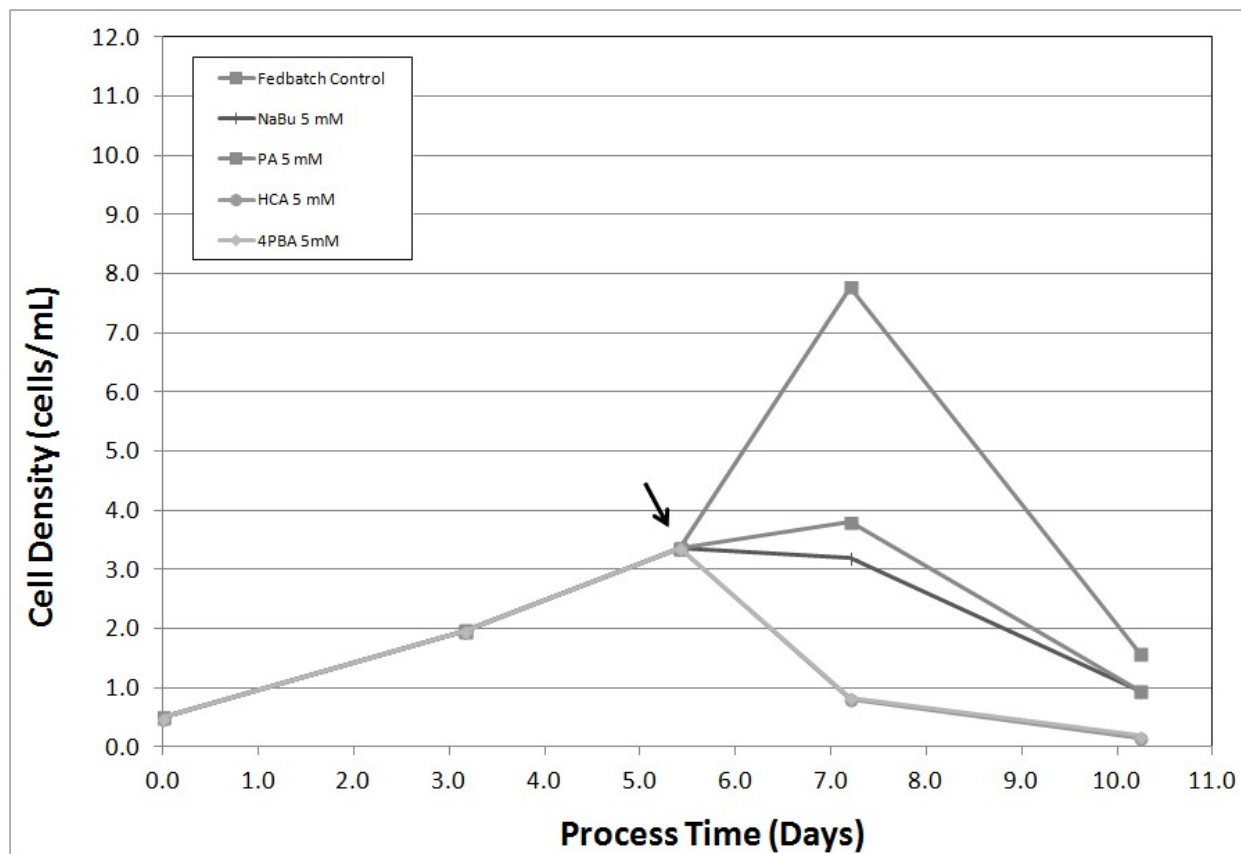
Figure

 11 Growth promotion of 5mM concentrations components causing reduced performance.

The largest volumetric productivity increase was observed in the 5mM Sodium Butyrate treatment condition, 72.01 mg/L (Table 1). However, Pentanoic acid showed a loss of volumetric productivity at 2.5 mM and 5.0 mM treatment levels. The peak volumetric productivity at 1.25 mM Pentanoic acid was 50.93 mg/L which was only 70.7% of the peak volumetric productivity of Sodium Butyrate at 5mM.

Table 5 Result of gradient testing of select SME and the antioxidant NAC.

		SME Conc (mM)	ICA (cell-days/mL) Integral Cell Area	tPA (mg/L) Volumetric Productivity	X' (pg/cell/day) Specific Productivity
Fed batch Control	Control	0.000	34.05	48.03	1.41
NAC 2.5 mM	NAC	2.500	45.25	62.19	1.37
NaBu 5 mM	NB	5.000	22.04	72.01	3.27
PA 1.25 mM	PA	1.250	34.56	50.93	1.47
HCA 0.625 mM	HCA	0.625	36.70	43.14	1.18
4PBA 0.625 mM	4PBA	0.625	26.80	24.74	0.92

Considering the integral cell area of treatment conditions, n-Acetyl-Cysteine showed decline in culture performance at the 10mM level compared to the 5mM level, 31.21 cell-days/mL compared to 45.83 cell-days/mL, respectively, a 31.9% drop. Peak volumetric productivity for n-Acetyl-cysteine, 62.19 mg/L was observed at 2.5 mM which was 64.2% higher than that observed at the peak cell performance level at the 5mM concentration.
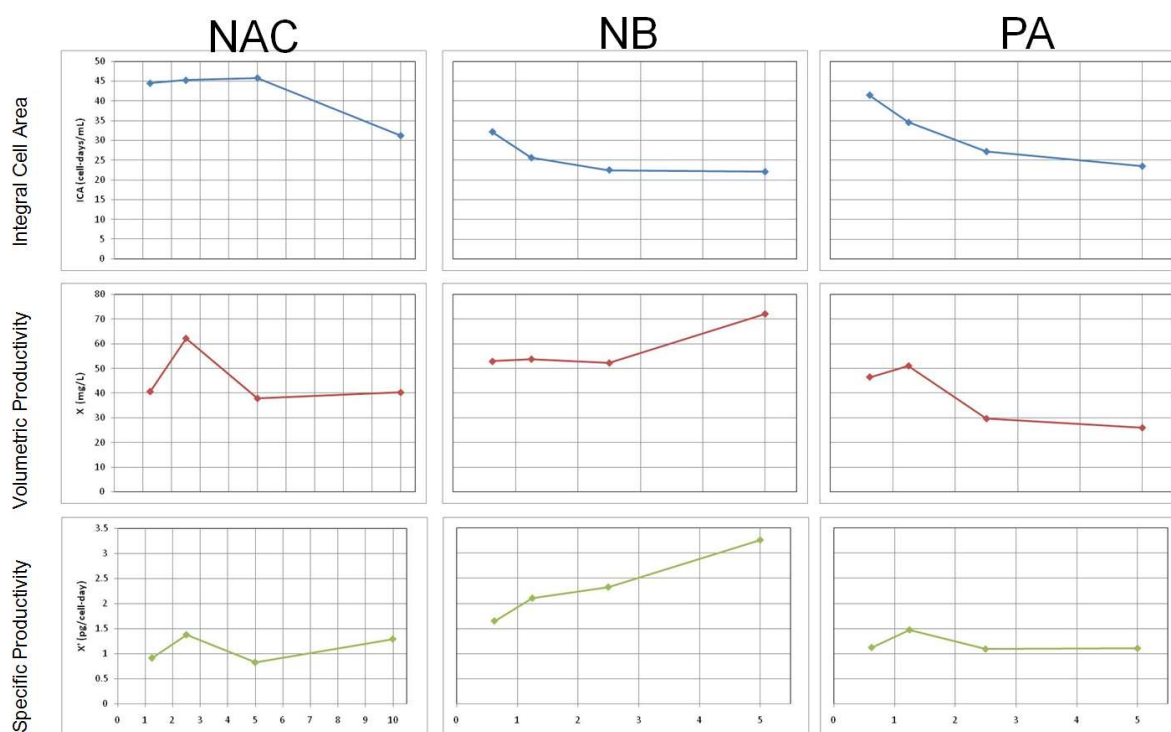
Figure 12 Plots of Integral Cel lArea , Viability, and Specific Productivity for n-Acetyl Cysteine (NAC), Sodium Butyrate (NB), and Pentanoic Acid (PA) condictions.
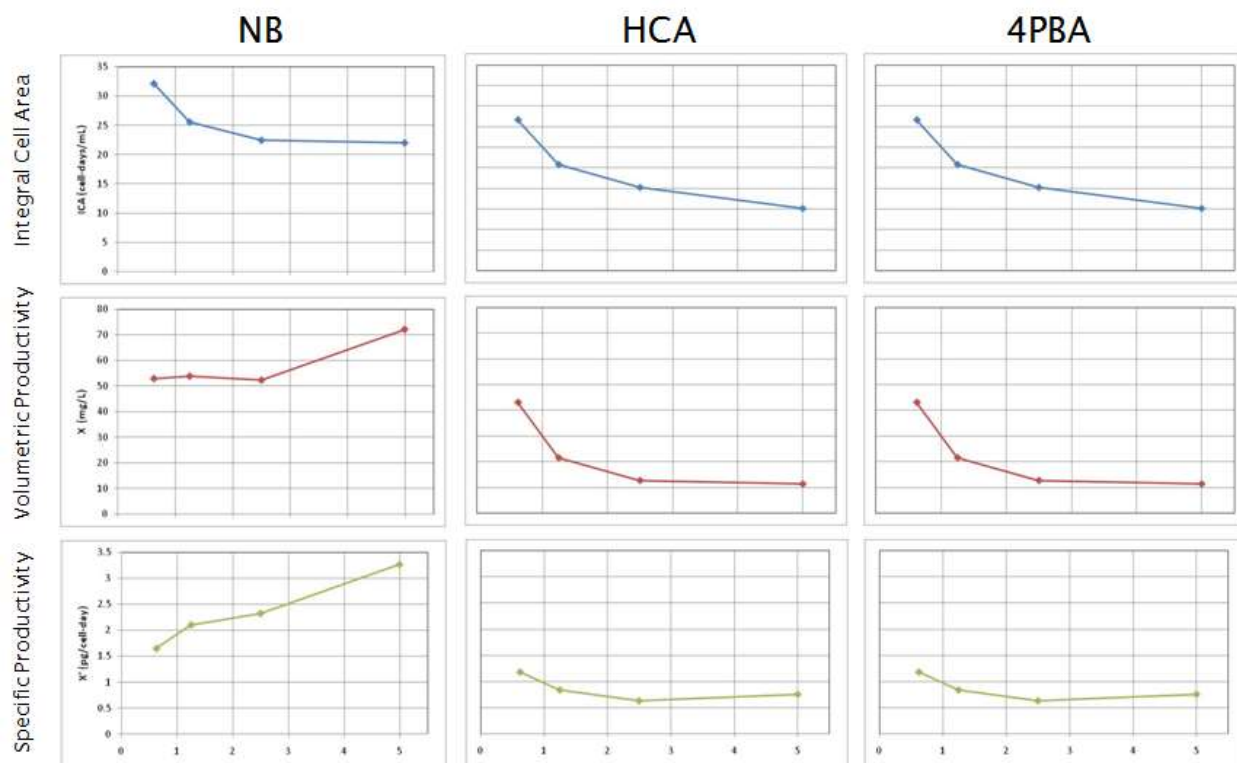
Figure 13 Plots of Integral CellArea , Viability, and Specific Productivity for Sodium Butyrate (NB), Hydrocinnamic Acid (HCA), and 4-Phenylbutanoic Acid (4PBA).

3.4   Conclusion

Multiple small molecules were demonstrated to show gradient dependent response to cell growth and protein production. In this tPA producing cell line the best protein production was seen in cultures induced with butanoic acid. The short-chain fatty acid pentanoic acid was able to improve productivity compared to the fed-batch control, but to a lower level than butanoic acid. The antioxidant, n-acetyl cysteine, was the only molecule evaluated in the test to show a marked improvement in cell densities over the testing gradient.

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Chapter

IMPROVED GROWTH AND RECOMBINANT PROTEIN PRODUCTIVITY IN CHO

CELLS USING THE COMBINED EFFECTS OF SHORT CHAINFATTY ACIDS WITH

THE ANTIOXIDANT N-ACETYLE CYSTEINE

4.1   Introduction

Within biopharmaceuticals, monoclonal antibodies are an important segment. Seven of

the top ten selling biopharmaceuticals in 2011 were monoclonal antibodies or fragments of

monoclonal antibodies. Monoclonal antibodies are natural occurring glycosylated proteins that

play a major role the body's immune system. They are monospecific to an epitope target and are

produced by B lymphocytes, a class of immune cells, in response to an antigen. Specific B

lymphocytes only produce one specific antibody. One method for producing the B cell needed

for the fusion is to expose a mouse to an antigen in two phases, a priming dose and a booster

dose. Following treatment with the antigen a sample of the spleen can be harvested. In 1975,

George Kohler and Cesar Milstein demonstrated that a specific B cells could be isolated and

fused with a cancerous myeloma cell line. The B cell bringing the ability to produce a specific

antibody and the myeloma cell conferring the ability to replicate rapidly and indefinitely. This

process, known as hybridoma technology, ushered in a process to allow expanded production of

a monoclonal antibody to a specific target molecule. Following fusion with the myeloma cell

line the hybridoma population can be screened for a population producing the antibody of

interest.

Most therapeutics monoclonal antibodies are either humanized or are human

immunoglobulins (IgG) and are produced as recombinant glycoproteins in mammalian cells

(Beck et al. 2008). Despite their rapid growth in use, they are generally expensive therapies that can cost over $100,000 per year or over the course of full treatment (Bioplan Associates 2013). The high cost of MAb treatment can be attributed to low potency and short half-life, requiring large dosages and frequent dosages. These factors lead to large amounts of MAb being needed to treat patient populations. It is estimated that around 90% of the world-wide production capacity for mammalian cell culture is for MAb production (Bioplan Associates 2013). Much of this production requires the use of large volume bioreactors at the 10,000 L scale. During process development and scale-up one of the primary engineering objectives is to optimized the production process to minimize manufacturing costs.

In an effort to target optimization of recombinant protein production of IgG in CHO cell lines we sought to analyze the growth and recombinant protein production response to the novel combination of pentanoic acid induction of protein production and the combined treatment with the anti-oxidant n-Acetyl Cysteine to minimize detrimental response by the cultured cells to treatment with the carboxylic acids.

4.2   Materials and Methods

A Chinese Hamster Ovary cell line producing a monoclonal antibody (IgG) was used for these studies. Seed stocks of the previously suspension adapted cell line were brought out of cryopreservation and were cultured with CDM4CHO® (HyClone, Thermo Fisher Scientific, Waltham, Massachusetts). This cell stock was cultured in volume of 100 mL in 250 mL disposable Erlenmeyer shake flasks in a 37°C, 5%CO_2 incubator, passaged every 3 to 4 days. Cultures were agitated on a shaker platform at 135 RPM with loosened caps to maintain adequate gas exchange.

4.2.1 Cell Line and Media

A Chinese Hamster Ovary cell line producing a monoclonal antibody (IgG) was used for these studies. The cell line was cultured with CDM4CHO® (HyClone, Thermo Fisher Scientific, Waltham, Massachusetts). Cell stocks were maintained in 200 mL shake flask cultures in a 37°C, 5%CO_2 incubator, passaged every 3 to 4 days.

4.2.2 Component Preparation

Butanoic Acid and pentanoic acid were prepared at a concentration of 250mM in deionized water. N-Acetyl –cysteine was prepared at a concentration of 500mM in deionized water.

4.2.3 Bioprocess Experiments

A time course, repeated measure study of the two short chain fatty acids, pentanoic acid and butanoic acid, were studied independently with n-acetyl cysteine in 5 x 5 factorial designs. Pentanoic acid and butanoic acid were run from 0 – 5 mM in a two-fold serial dilution gradients and n-acetyl cysteine was run from 0 – 10 mM in two-fold serial dilution gradients. The samples were randomized and run in triplicate. Conditions were 1.0 mL cultures run in deep-well 96 well microtiter plates (Nunc, Thermo Fisher Scientific, Waltham, Massachusetts).

Experimental conditions were cultured in a basal formulation of CDM4CHO® supplemented with 6 g/L Cell Boost 2® (HyClone, Thermo Fisher Scientific, Waltham, Massachusetts) process supplement. Cultures were maintained in a 37°C, 5%CO2 incubator active humidification at a relative humidity of 80%. Mixing was provided by orbital shaking at

1,000 RPM with a 3mm orbit. Culture sterility was maintained using rayon fabric covers to seal the plates.

All culture conditions were inoculated at 250,000 cells/mL on day 0 and were induced with butanoic acid or pentanoic acid on day 4. Supplementation of culture with NAC was done at the time of small molecule induction. Cultures were sampled on days 0, 3, 5, 7, and 10 for cell quantitation and product titer.

4.2.4   Cell Quantitation

Cell density and viability were determined using. Trypan blue, a dead-cell dye, excluded by viable cells was used to distinguish the two different cell populations. A high throughput image cytometer, Cellavista ® Cell Imager (SynenTec, Elmshorn, Germany), was used to count microtiter plate samples of culture conditions. Aliquots for cell counting were counted immediately after sampling.

4.2.5   Product Titer Analysis by Biolayer Interpherometry

Samples of 40uL from culture conditions were diluted in phosphate buffered saline (PBS) in standard 96 well SBS microtiter plates and were kept frozen at -20°C until analysis. Product titer was determined using a biolayer interpherometry assay with Protein A biosensors using an Octet QK384 (Fortebio, Menlo Park, CA). A reference standard was generated using a human IgG (Sigma Aldrich, St. Louis, MO) in the range of 3.16 to 1000 ug/mL.

4.3   Results and Discussion

4.3.1   Effects of Butanoic and Pentanoic Acids in combination with N-Acetyl Cysteine on Cell Growth Profiles Density

The treatment of a CHO cell line producing a recombinant IgG with small molecule enhancers and the anti-oxidant n-acetyl cysteine was analyzed in a scale-down model using deep-well microtiter plates. The cultures demonstrated expected growth response (Figure14) to treatments. Significant loss in viable cells was observed in cultures treated with higher concentrations of small molecule enhancers. Loss of viable cells was observed in cultures treated with higher concentrations of small molecule enhancers. Higher peak growth was observed in cultures treated with pentanoic acid relative to cultures treated with butanoic acid (Figure 16).
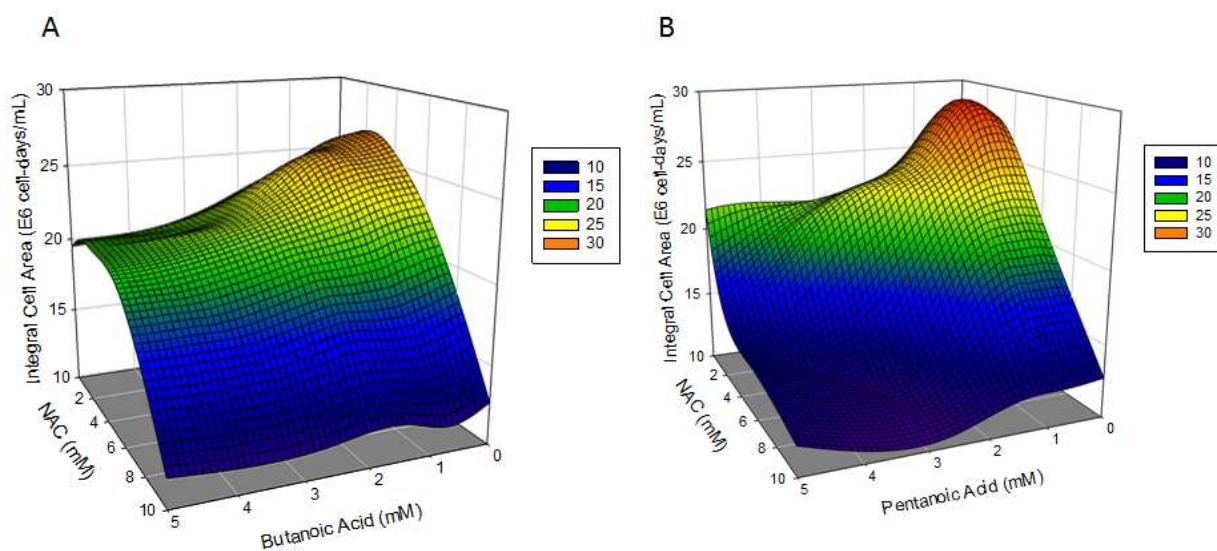
Figure 14 Response Surface growth promotion study. (A) Butanoic acid and n-acetyl cysteine. (B) Pentanoic acid and n-Acetyl Cysteine.

Butanoic acid induction in combination with NAC, in a high throughput response surface methodology design, at a concentration of 0.625 mM butanoic acid and 1.25 mM n-acetyl cysteine, had a 6.2% increase in growth (Figure14A) compared to the untreated control. At the same concentration of butanoic acid, but no NAC supplementation the growth was decreased by

34.6% compared to the non-treated control compared to the 1.25mM NAC treated cultures at the same butanoic acid concentration.

Pentanoic acid induction in combination with NAC, at a concentration of 0.625 mM pentanoic acid and 1.25 mM n-acetyl cysteine, had a 16.6% increase in growth (Figure14B) compared to the untreated control. At the same concentration of pentanoic acid, but no NAC supplementation the growth was increased by 16.6% compared to the non-treated control and compared to the 1.25mM NAC treated cultures at the same pentanoic acid concentration.

Higher peak growth was observed in cultures treated with pentanoic acid relative to cultures treated with butanoic acid. Interestingly, there was improvement in growth of CHO cultures treated with pentanoic acid relative to the untreated control sample, but this wasn't observed in butanoic acid treated samples (Figure 15).
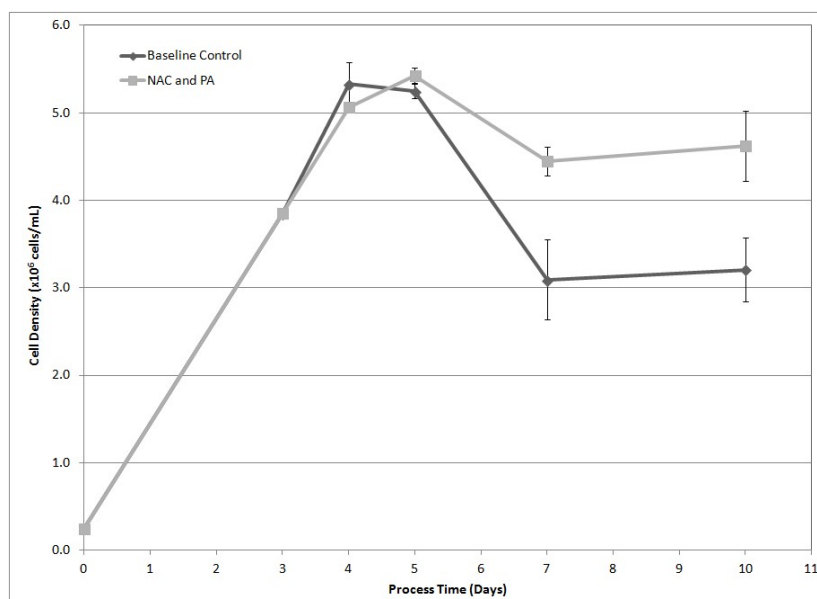
Figure 15 Plot of growth performance of CHO cells with combined Pentanoic Acid (PA) and n-Acetyl Cysteine treatment (NAC) compared to untreated control.

4.3.2   Effects of Butanoic and Pentanoic Acids in combination with N-Acetyl Cysteine on

        Volumetric Productivity

Comparison of the treatment of cultures with various levels of pentanoic acid and

butanoic acid with the addition of anti-oxidant didn't show major differences in volumetric

productivity (Figure 16), but did show variation in concentration for peak productivity. Peak

productivity was observed for butanoic acid and NAC at 0.625 mM and 1.25 mM, respectively.

Peak productivity was observed for pentanoic acid and NAC at 1.25 mM and 1.25 mM,

respectively.
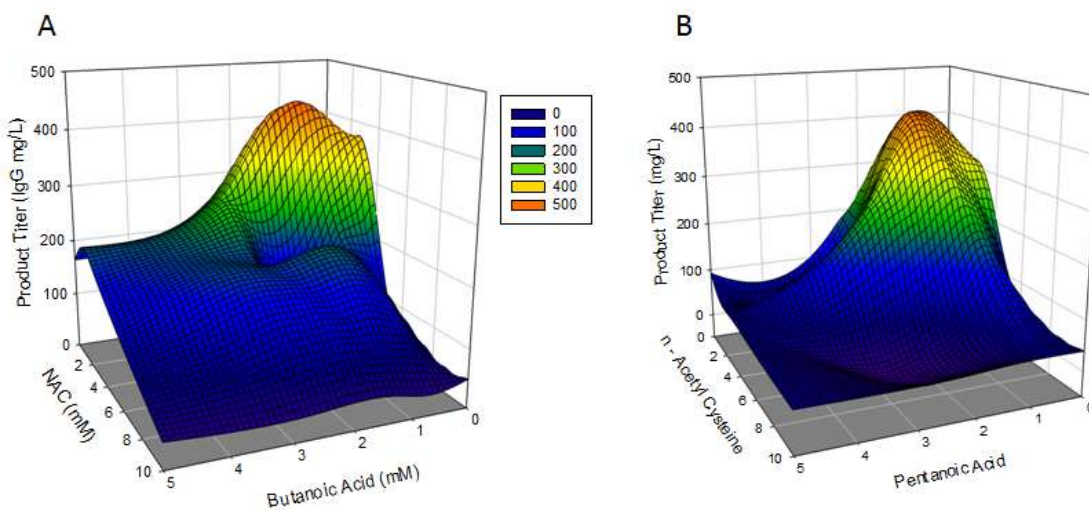
Figure

 16 Response Surface of production of IgG. (A) Butanoic acid and n-acetyl cysteine. (B) Pentanoic acid and n-Acetyl Cysteine.

Butanoic acid induction in combination with NAC, in a high throughput response surface

methodology design, at a concentration of 0.625 mM butanoic acid and 1.25 mM n-acetyl

cysteine, a 3.52 factor increase in production of IgG (Figure 17) compared to the untreated control. At the same concentration of butanoic acid, but no NAC supplementation produced 87.3% less IgG compared to the 1.25mM NAC treated cultures at the same butanoic acid concentration.

Pentanoic acid induction in combination with NAC, in a high throughput response surface methodology design, at a concentration of 1.25 mM pentanoic acid and 1.25 mM n-acetyl cysteine, a 3.68 factor increase in production of IgG (Figure 18) compared to the untreated control. At the same concentration of pentanoic acid, but no NAC supplementation produced 55.7% less IgG compared to the 1.25mM NAC treated cultures at the same pentanoic acid concentration.
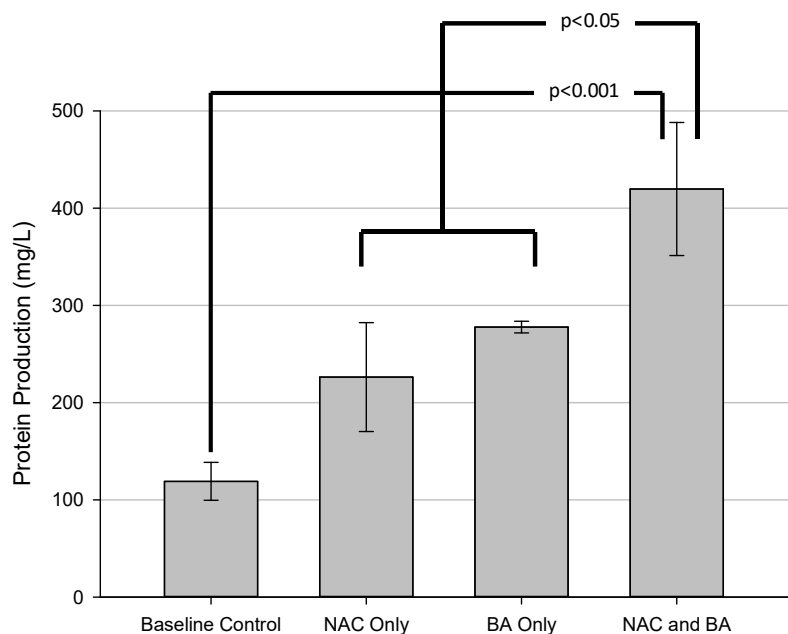
Figure17 Butanoic Acid and n-Acetyl Cysteine treatment.
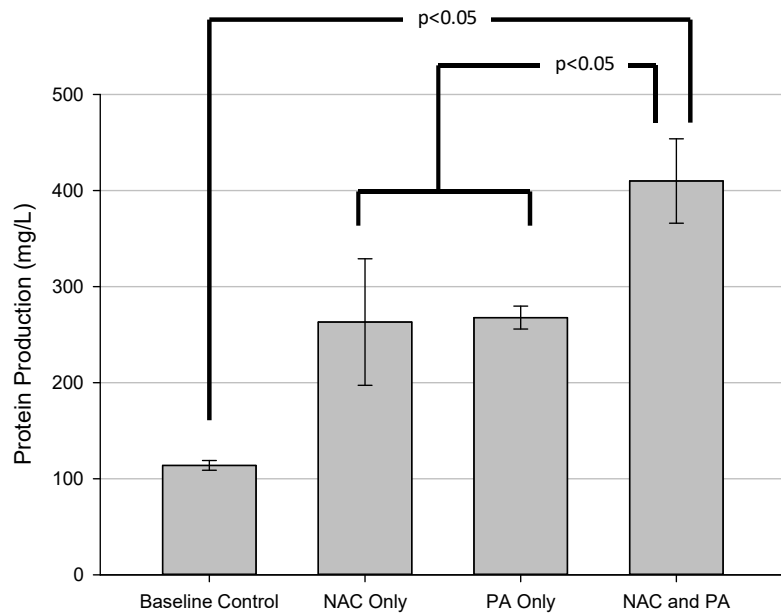
Figure18 Pentanoic Acid and n-Acetyl Cysteine treatment.

Conclusion

Using the antioxidant n-acetyl cysteine we have shown improved production of IgG in a

CHO cell line cultured with pentanoic acid used as an enhancer of protein production. The

improvement in productivity with the combined treatment of pentanoic acid and n-acetyl

cysteine correlated with improved viability. Co-supplementation of the two molecules may be

an effective tool for use in optimization of cell culture bioprocesses. Butanoic acid and

pentanoic acid can induce increased production of recombinant IgG in CHO cells.

Supplementation of n-acetyl cysteine to pentanoic acid induced cultures can further increase IgG

production increases.

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Chapter

DECREASED APOPTOTIC EFFECT USING THE ANTIOXIDANT N-ACETYLE CYSTEINE IN COMBINATION WITH TREATMENT OF CHO CELLS WITH THE SHORT CHAIN FATTY ACIDS BUTANOIC ACID AND PENTANOIC ACID

5.1   Introduction

Tissue plasminogen activator (tPA) is a 68 kDa monomeric serine protease (EC 3.4.21.68) produced primarily by endothelial cells. In blood, it is one of two major activators of plasminogen, the precursor of plasmin which is a serine protease that can dissolve fibrin blood clots. A recombinant form of the protein is produced as a biopharmaceutical drug, Activase®, for treatment of acute myocardial infarction, acute ischemic stroke, pulmonary thrombosis, and other blood clot conditions (Wagstaff et al. 1995). It is a recombinantly produced protein and is made in CHO cells and acts as a model for non-IgG biopharmaceutical recombinant protein production systems.

5.2   Materials and Methods

5.2.1   Cell Line and Media

A Chinese Hamster Ovary cell line, CHO 1-15$_{500}$ (ATCC® CRL-9606™), producing human tissue Plasminogen Activator (tPA) was used for these studies. The cell line was cultured with CDM4CHO® (HyClone, Thermo Fisher Scientific, Waltham, Massachusetts). Cell stocks were maintained in 200 mL shake flask cultures in a 37°C, 5%CO_2 incubator, passaged every 3 to 4 days.

Experimental conditions were cultured in CDM4CHO® supplemented with 2 g/L Cell Boost 2® (HyClone, Thermo Fisher Scientific, Waltham, Massachusetts) process supplement.

5.2.2 Component Preparation

Butanoic Acid was prepared at a concentration of 250mM in deionized water. Pentanoic acid was prepared at a concentration of 250mM in deionized water. N-Acetyl Cysteine was prepared at a concentration of 500mM.

5.2.3 Bioprocess Experiments

Disposable shake flasks were used for 50 mL scale-down culture testing of butanoic acid and pentanoic acid with n-Acetyl Cysteine supplementation for studying apoptosis and productivity.

Disposable 125 mL Erlenmeyer shake flasks were used for 50 mL scale-down culture testing of butanoic acid and pentanoic acid with n-acetyl cysteine supplementation for studying apoptosis and productivity. Cultures were maintained in a non-humidified 37°C, 5%CO2 incubator. Mixing was provided by an orbital shaker, shaken at 135 RPM with a 19 mm orbit.

All culture conditions were inoculated at 250,000 cells/mL on day 0 and were induced with butanoic acid or pentanoic acid on day 3. Supplementation of culture with NAC was done at the time of small molecule induction of cultures. Cultures were sampled on days 0, 3, 4, 5, 6, 7, and 10 for cell quantitation and product titer. Apoptosis was analyzed on day 6, three days post induction with butanoic acid and pentanoic acid.

5.2.4 Cell Quantitation

Cell density and viability were determined using Trypan Blue dye exclusion assays. Microtiter plate cultures were counted using on a Cellavista® Cell Imager (SynenTec, Elmshorn,

Germany). Shaker flask cultures were counted using an automated ViCell® XR Cell Viability

Analyzer (Beckman Coulter, Fullerton, California). Aliquots for cell counting were counted

immediately after sampling.

5.2.5   Apoptosis Analysis by Flow Cytometry

Apoptosis, genetically programmed cell death, is a natural response for removal of

unwanted cells (Elmore 2007). The process of programmed cell death is characterized by

multiple cellular events; chromatin condensation, cell volume loss, DNA fragmentation, and the

loss of phospholipid membrane integrity. The loss of phospholipid membrane integrity results in

phosphatidylserine (Bratton et al. 1997) exposure on the extracellular cell membrane.

Phosphatidylserine, a negatively charged phospholipid component of the inner cell membrane,

when exposed on the outer cell membrane signals recognition of the of the apoptotic cells

initiating a non-inflammatory phagocytic response.

Annexins are a family of proteins that bind to cell membranes. Annexin V binds with

high specificity to phosphatidylserine. The binding takes place rapidly with high affinity and is

dependent on calcium for binding.

Apoptosis was analyzed following treatment of cells with butanoic acid or pentanoic acid

combined with treatment by n-Acetyl Cysteine. Early apoptosis was monitored by

phosphatidylserine exposure on the cell surface using an fluorescein isothiocyanate labeled

Annexin V (Vermes et al. 1995). To distinguish dead cells from apoptotic cells propidium

iodide was used to label dead cells. Propidium iodide is a fluorescent molecule that intercalates

in dead cell and binds with nucleic acids. Annexin V, propidium iodide treated cells were

analyzed using a BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, CA) with a

HyperCyt® Autosampler (Intellicyt Corporation, Albuquerque, NM).

5.2.6   Product Titer Analysis by Enzyme-Linked Immunosorbent Assay

Product titer was determined using a sandwich enzyme-linked immunosorbent assay

(ELISA). An affinity purified sheep anti-tPA IgG was used as a capture antibody by coating a

standard ELISA 96 well plate. A reference standard curve was generated using a concentrated

and titered recombinant tPA control in the range of 3.16 to 1000 ug/mL. Samples from culture

conditions which had previously been stored frozen at -20°C were thawed and dilutions were

prepared in phosphate buffered saline (PBS) at a dilution factor of 1:2000. Sample were then

applied to the capture antibody prepared plates. A sheep anti-tPA conjugated to horse-radish

peroxidase was used as the detecting antibody. A stable liquid one-step 3,3′,5,5′-

tetramethylbenzidine (TMB) liquid was used as a substrate for colorimetric analysis. A

SpectraMax M2 (Molecular Devices, Sunnyvale, CA) was used to analyze the assay plates at a

detection wavelength of 450 nm.

5.3   Results and Discussion

The addition of the anti-oxidant n-Acetyl Cysteine to the culture of CHO cells producing

a recombinant protein ameliorated the apoptotic effect of carboxylic acid treatment with butanoic

acid and pentanoic acid.

5.3.1   Effects of Butanoic Acid and Pentanoic Acid in combination with N-Acetyl Cysteine on

        Apoptosis

The treatment of CHO IgG cell line producing a recombinant IgG1 was cultured in small

volume shake flasks.   Cultures were treated with different levels of butanoic acid, pentanoic

acid, and the anti-oxidant n-acetyl cysteine (NAC) on day 3. On day 7 samples were analyzed

for apoptosis using Annexin V and propidium iodide with analysis by flow cytometry. Annexin

V was used identify cells that were entering early apoptosis and propidium iodide was used to

identify cells that had died. The control culture had a viability of 86.9% (Table 6) and showed

normal growth characteristics.
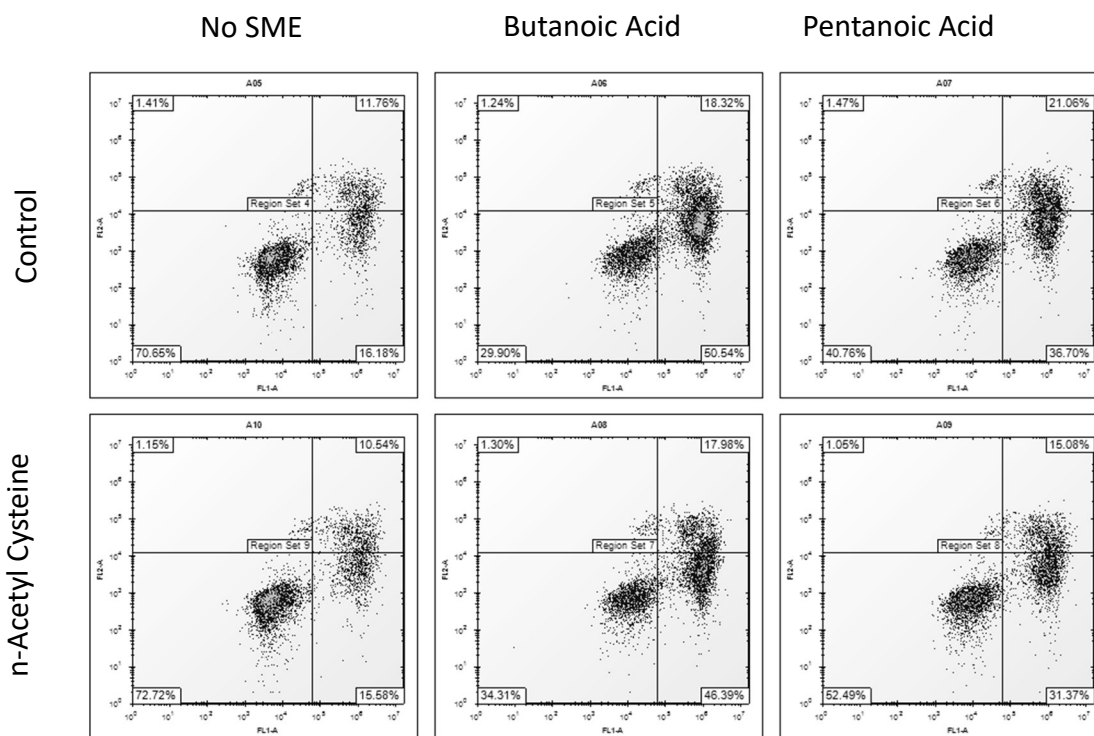
Figure 19 Flow cytometry analysis of carboxylic acid treatment with the antioxidant n-Acetyl Cysteine compared to an untreated control.

Table 6 Viability of combined carboxylic acid and antioxidant treatment.

Viable%	No SME	Butyrate 0.625 mM	Pentanoate 0.625 mM
0 mM NAC	86.9%	80.4%	77.5%
1.25 mM NAC	88.3%	80.7%	83.9%

Table 7 Early Apoptosis results of combined carboxylic acid and antioxidant treatment.

Early Apoptosis%	No SME	Butyrate 0.625 mM	Pentanoate 0.625 mM
0 mM NAC	19.0%	62.8%	47.4%
1.25 mM NAC	17.7%	57.5%	37.4%

The culture treated with n-acetyl cysteine only showed minimal improvements in culture viability and cells not entering early apoptosis (Table 6), 1.4% and 1.3%, respectively. Treatment of cultures with sodium butyrate and NAC showed minimal improvement in viability but improved the percentage of cells entering early apoptosis by 5.3% (Table 6).   Pentanoic acid and NAC treated culture demonstrated 6.4% improvement in viability and 10.0% improvement in preventing cells from entering early apoptosis (Table 7). All conditions showed improved productivity relative to the untreated control (Table 8). In this study butanoic demonstrated a 5.7% loss in productivity when treated with NAC, however, pentanoic acid treatment with NAC demonstrated an improvement in productivity of 19.5% (Figure 8).

5.3.2   Comparison of Viability, Early-Apoptosis and Productivity Improvement

Treatment of pentanoic acid containing culture with n-acetyl cysteine demonstrated a 19.5% increase in productivity and a 7.3% increase in viability (Table 8). A reduction of 5.72% in the amount of IgG produced was observed in butanoic acid cultures treated with the antioxidant n-acetyl cysteine. Reduced levels of apoptosis were observed in cultures of both pentanoic acid and butanoic acid.

Table 8 Productivity results of combined carboxylic acid and antioxidant treatment.

Productivity (Relative to Control)	No SME	Butyrate 0.625 mM	Pentanoate 0.625 mM
0 mM NAC	1.0	2.56	1.44
1.25 mM NAC	1.12	2.42	1.71

5.4   Discussion

Butanoic acid is a well-known small molecule enhancer of protein production, but induces apoptosis in many different cell types. n-Acetyl Cysteine is a thiol containing anti-oxidant that has been shown to reduce apoptosis induction by butanoic acid. Pentanoic acid has been shown to have similar properties for inducing higher production levels of proteins. We have been able to demonstrate that pentanoic acid can improve protein production.   It also shows increased apoptosis, which can be delayed by treatment with n-acetyl cysteine.

Here it was shown that pentanoic acid can improve the volumetric production of IgG in CHO cells treated during log phase growth. This stimulation was positive but didn't achieve the productivity levels achieve with butanoic acid supplementation. Pentanoic acid experiments showed similar improvements in productivity to those observe by Liu et al. (Liu et al. 2001c).

We were able to show apoptosis markers in conditions treated with both pentanoic acid and butanoic acid. Although the supplementation of the anti-oxidant n-Acetyl Cysteine into both treatments showed improvements in cell health, only pentanoic acid showed improvement productivity. We did observe further performance increases to those previously observed by others for increased productivity with the addition of anti-oxidants (Oh et al. 2005). However, the overall volumetric productivity was 1.7-fold higher in butanoic acid treated cultures.

5.5   Conclusion

As seen in previous studies, pentanoic acid wasn't able to stimulate higher productivity than butanoic acid.   However, the combined treatment with the antioxidant n-acetyl cysteine was able to improve productivity in pentanoic acid treated cultures to a greater degree, than butanoic acid treated conditions. Apoptosis reduction by treatment with n-acetyl cysteine in cultures induced with carboxylic acid was shown to be a viable method to improve productivity.

5.6   References

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Chapter

SUMMARY, CONCLUSIONS, AND RECOMMONDENDATIONS

6.1   Summary and Conclusions

In an effort to optimize the bioprocess manufacturing of recombinant proteins by increasing the volumetric titer, the combination for protein induction of the short chain fatty acid pentanoic acid and the antioxidant n-acetyl cysteine is a viable method for process evaluation. The improvement in productivity with the combined treatment correlated with improved viability and a reduction in cells entering apoptosis. Butanoic acid and pentanoic acid were both shown to induce increased production of recombinant IgG in CHO cells. The levels of improved protein production were shown to be cell line dependent for both of these carboxylic acids.

6.2   Recommendations

A. Perform scale-up testing of scale-down testing from research. In these studies, scale-down models of larger bioprocess systems were used. Benchtop bioreactors allow the testing of the samples under more controlled conditions, specifically of control dissolved oxygen (DO) and pH conditions. These factors being held at a steady state level allow optimization to add in specific parameter controls at targeted levels.

B. Study in chemostat culture would allow for steady analysis looking at changes in oxygen consumption, glucose consumption, lactic acid production, protein production, death rate and growth rate under various steady-state conditions.

C. Investigate effect of genetic modification of the cell line, by such things as bcl-2 overexpression with addition of various small molecule enhancers of production.

D. Test mechanism of action of PA and other SME for HDAC activity.

E. New clones are invariably different, so design and development of standardized screening process can allow rapid identification of optimization pathways. Other cell lines such as CHO1-15, non-IgG producers

F. SME. Perform broad screening assays of small molecule enhancers of protein productivity. This can be achieved by development of high throughput screening assays under appropriate culture conditions. Molecule that could be screened are short-chain fatty acids (SCFA), including butanoic acid and pentanoic acid, aromatic carboxylic acids, hydroxamic acids, acetamides, DMSO, rapamycin, etc. Screening can narrow down the scope of analysis allowing best performers to proceed to additional testing.

G. Antioxidants. Develop a high throughput screening assay of a broad spectrum of antioxidants. Look at antioxidants such as what are the classes of antioxidants.

H. Combined effect of antioxidants and SME's. Look at different screening designs to determine if their combinations of SME may act synergistically to improve productivity.

I. Analyze small molecule enhancers effect on cell cycle. Is there a particular supplement that will improve the cell survival of samples in the G0 cell cycle?

J. Look at critical quality attributes. Study the glycosylation pattern of SME treated and AOx treated. Glycosylation deamidation. Protein aggregates. Bioactivity. Look at Sialic acid content of antibodies. Maybe Lectin binding. Look at protein aggregation, sequence truncation, and charge variants. Determine if there is a time-coarse variation in change of critical quality attributes. (Sung and Lee 2005) Final product activity assay.

K. Focus comparison of feeding strategies effect on induction. Low temperature shift combined affect. Analyze effect of SME addition rate of apoptosis. Rather than a bolus

induction, could a slow addition improve effect while minimizing negative effects like apoptosis.

L. Analyze intracellular igg versus expression for potential that over production may be cause lower levels Analysis of transcription and translation mRNA levels

6.3   References

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APPENDIX

APPENDIX A STUDY DESIGN/SAMPLING

7.1   Apoptosis Analysis - Annexin V/Propidium Iodide

Apoptosis is a process of programmed cell death, initiated by stimulation of signaling cascade. The monitoring of apoptosis offers a tool in biological discovery process. One method for monitoring apoptosis is the use Annexin V, that can be bound to a fluorescent molecule such as fluorescein isothiocyanate (FITC). This molecule can selectively bind to the cell surface protein phosphatidyl-serine. Phosphatidyl-serine is typically attached to the interior of cell membrane. During apoptosis, prior to the loss of cell membrane integrity, phosphatidyl-serine will shift to the extracellular side of the cell membrane. This process acts as a marker for early apoptosis. Staining with Annexin V is generally combined with a vital dye such as propidium iodide (PI) or with 7-Amino-Acintomycin (7-AAD). PI and 7-AAD are able to stain dead and damaged cells because their membrane is permeable. Cells not staining Annexin V and not for PI are considered viable and non-apoptotic, those staining for Annexin V but not PI are considered to be early apoptotic, and those staining for both dyes are considered in late apoptosis or dead. Using a combination of these dyes is not able to distinguish between cells that have died from apoptosis or necrosis. However, measuring the cell population over time, one can see the progression of the cell population from viable, living cells to early apoptotic cells, to late stage apoptosis and death.

7.2   Staining Protocol

Wash cells twice with cold PBS and then resuspend cells in 1X Binding Buffer at a concentration of 1 x 10^6 cells/ml.

Transfer 100 μL of the solution (1 x 10^5 cells) to a 5 ml culture tube.

Add 5 μL of FITC Annexin V and 5 μL PI.

Gently vortex the cells and incubate for 15 min at RT (25°C) in the dark.

Add 400 μL of 1X Binding Buffer to each tube. Analyze by flow cytometry within 1 hr.

APPENDIX B SUPPLEMENTARY DATA

Experiment 1

	SME Conc (mM)	ICA (x10^6 cell-days/mL) Integral Cell Area	tPA (mg/L) Volumetric Productivity	X' (pg/cell/day) Specific Productivity
NAC	10	31.21	40.28	1.29
NAC	5	45.83	37.87	0.83
NAC	2.5	45.25	62.19	1.37
NAC	1.25	44.49	40.67	0.91
NB	5	22.04	72.01	3.27
NB	2.5	22.48	52.27	2.32
NB	1.25	25.59	53.81	2.10
NB	0.625	32.13	52.89	1.65
PA	5	23.52	25.96	1.10
PA	2.5	27.21	29.64	1.09
PA	1.25	34.56	50.93	1.47
PA	0.625	41.43	46.43	1.12
HCA	5	15.08	11.35	0.75
HCA	2.5	20.20	12.71	0.63
HCA	1.25	25.81	21.57	0.84
HCA	0.625	36.70	43.14	1.18
4PBA	5	15.20	11.35	0.75
4PBA	2.5	15.71	11.35	0.72
4PBA	1.25	21.69	15.08	0.70
4PBA	0.625	26.80	24.74	0.92

Experiment 2

BA and NAC Protein Production

One-way Analysis of Variance (ANOVA)

BA ICA

Comparison	Mean Difference	q		P value
Control Baselin vs NAC Only	-1.845	7.099	**	P<0.01
Control Baselin vs BA Only	0.4610	1.587	ns	P>0.05
Control Baselin vs NAC and BA	-1.499	5.768	*	P<0.05
NAC Only vs BA Only	2.306	7.936	**	P<0.01
NAC Only vs NAC and BA	0.3460	1.331	ns	P>0.05
BA Only vs NAC and BA	-1.960	6.745	**	P<0.01

BA P

Comparison	Mean Difference	q		P value
Control Baselin vs NAC Only	-107.33	3.834	ns	P>0.05
Control Baselin vs BA Only	-158.75	5.073	*	P<0.05
Control Baselin vs NAC and BA	-300.83	10.747	***	P<0.001
NAC Only vs BA Only	-51.417	1.643	ns	P>0.05
NAC Only vs NAC and BA	-193.50	6.913	**	P<0.01
BA Only vs NAC and BA	-142.08	4.540	ns	P>0.05

BA q_p

Comparison	Mean Difference	q		P value
Control Baselin vs NAC Only	-3.740	3.457	ns	P>0.05
Control Baselin vs BA Only	-6.742	5.575	*	P<0.05
Control Baselin vs NAC and BA	-11.367	10.508	***	P<0.001
NAC Only vs BA Only	-3.002	2.482	ns	P>0.05
NAC Only vs NAC and BA	-7.627	7.051	**	P<0.01
BA Only vs NAC and BA	-4.625	3.824	ns	P>0.05

Experiment 2

PA and NAC Protein Production

One-way Analysis of Variance (ANOVA)

PA ICA

Comparison	Mean Difference	q		P value
Control Baselin vs NAC Only	-2.650	12.063	***	P<0.001
Control Baselin vs PA Only	0.4200	1.912	ns	P>0.05
Control Baselin vs NAC and PA	-1.350	6.145	*	P<0.05
NAC Only vs PA Only	3.070	13.975	***	P<0.001
NAC Only vs NAC and PA	1.300	5.918	*	P<0.05
PA Only vs NAC and PA	-1.770	8.057	**	P<0.01

PA P

Comparison	Mean Difference	q		P value
Control Baselin vs NAC Only	-149.34	6.451	**	P<0.01
Control Baselin vs PA Only	-154.00	6.652	**	P<0.01
Control Baselin vs NAC and PA	-296.17	12.793	***	P<0.001
NAC Only vs PA Only	-4.660	0.2013	ns	P>0.05
NAC Only vs NAC and PA	-146.83	6.342	**	P<0.01
PA Only vs NAC and PA	-142.17	6.141	*	P<0.05

PA q_P

Comparison	Mean Difference	q		P value
Control Baselin vs NAC Only	-5.061	6.125	*	P<0.05
Control Baselin vs PA Only	-6.506	7.874	**	P<0.01
Control Baselin vs NAC and PA	-11.252	13.618	***	P<0.001
NAC Only vs PA Only	-1.445	1.749	ns	P>0.05
NAC Only vs NAC and PA	-6.191	7.493	**	P<0.01
PA Only vs NAC and PA	-4.746	5.744	*	P<0.05