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Engineering Oxidative Stress Resistance in CHO Cell Factories

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biological Engineering

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"The true sign of intelligence is not knowledge, but imagination."

- Albert Einstein

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Abstract

Oxidative stress is a phenomenon created by an imbalance in the amount of Reactive Oxygen Species (ROS) created within a cell, and the ability of its defence mechanisms to effectively deal with ROS. Oxidative stress is extremely deleterious to the cell, and is known to cause damage to DNA, proteins and lipids (*Turrens, 2003*). Mitochondria are the cell's predominant producer of ROS (*Murphy, 2009*), but it has also been shown that increased protein folding in the Endoplasmic Reticulum (ER) results in an increase in ROS levels (*Malhotra, 2008*), an issue particularly pertinent as developers move towards hard-to-express proteins. As well as many enzymes dedicated to the eradication of ROS, such as caspases, peroxidases and superoxide dismutases (SODs) the cell maintains a glutathione pool to buffer the increase of ROS (*Lu, 2009*).

Design of Experiment models were designed and implemented using the growth, productivity and ROS content data from batch experiments in order to design anti-oxidant supplementation strategies. Two rounds of fed-batch screening were performed and a feeding strategy identified that improved the growth and ROS burden of three cell lines producing the same recombinant MAb product.

A directed evolution strategy was employed to engineer oxidative stress resistant host cell lines through chronic exposure to Hydrogen Peroxide. Following transfection with a recombinant MAb product, the novel engineered cell line consistently outperformed the original cell line in terms of growth and ROS content, in both transient and stable transfection processes. Doubling time of stably transfected evolved cell line was reduced to 23 hours, a substantial time frame reduction.

A link between ROS level reduction and improvement in cell line performance was demonstrated, with further investigation needed to unpick the mechanistic underpinnings of the oxidative stress resistance as well as to attempt to address the imbalance of improvements in growth compared to productivity.

Chapter 1:

Introduction

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List of Abbreviations

ROS	Reactive Oxygen Species
CHO	Chinese Hamster Ovary
UPR	Unfolded Protein Response
NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate
GSH	Glutathione

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1.1 The Importance of Anti-Oxidants

Should the reader type "anti-oxidant" into any online search engine query box, they would be confronted with a plethora of pharmacological and medical websites discussing the apparent health benefits of including anti-oxidants in everything from foods to skin care products, and exploring comparative successes or defeats in the use of anti-oxidants to treat various forms of cancer. Indeed, nutrition blogs often explain the numerous ways in which a health-conscious internet surfer can introduce these compounds into their diet and thus apparently improve everything from their chronic health conditions to the visible signs of ageing.

There is a high level of truth behind the hype. According to Mirriam-Webster's Learner's Dictionary, "anti-oxidant" is a term used to describe "a substance that inhibits oxidation or reactions promoted by oxygen, peroxides, or free radicals", and the reason for the high level of interest in their pharmaceutical benefits is due to the growing body of evidence that so-called Reactive Oxygen Species (ROS) such as Hydroxyl Radicals, Superoxide and Hydrogen Peroxide are causative factors in a wide range of diseases and illnesses ranging from debilitating neurological conditions such as Alzheimer's Disease and Parkinson's Disease (Liu *et* al, 1999) to milder diseases such as skin conditions (Bickers & Athar, 2006). ROS are elevated in almost all cancers, whilst cancer cells also have elevated intracellular defense mechanisms (Liou & Storz, 2010). Present in all mammalian cells, ROS are generated through processes such as energy production in oxidative phosphorylation (Muller *et al*, 2004) and protein folding and secretion (Malhotra *et al*, 2007) and while cells have developed signaling mechanisms incorporating ROS (Thannickal *et al*, 2000), intracellular build up is extremely deleterious to the cells, resulting in damage to DNA, lipids and proteins (Sies, 2015). Figure 1.1 gives an overview of ROS production within the cell and the various pathways and enzymes that exist to defend against accumulation of ROS and subsequent oxidative stress. Medical trials have been conducted investigating numerous anti-oxidants as therapeutics including cysteine precursors such as N-Acetylsycteine and Procysteine (Kim *et al*, 2014), scavenging compounds such as α-tocopherol, glutathione and α-lipoic acids (Packer *et al*, 2001; Vasdev *et al*, 2000) and mitochondrially targeted molecules such as MitoQ (Tauskela, 2007) and Szeto-Schiller Peptides (Li *et al*, 2011; Moreira *et al*, 2010).

1.2 Biopharmaceutical Production and Oxidative Stress

In 2014, the biopharmaceutical industry was worth a reported $163billion worldwide and made up roughly 20% of the total pharmaceuticals market (Otto *et al*, 2014). With mammalian cells accounting for the vast majority of production platforms within that industry, and CHO cells accounting for roughly 70% of these mammalian platforms, CHO cell factories used for biologics

production represent billions of dollars of revenue worldwide and continue to dominate the industry.

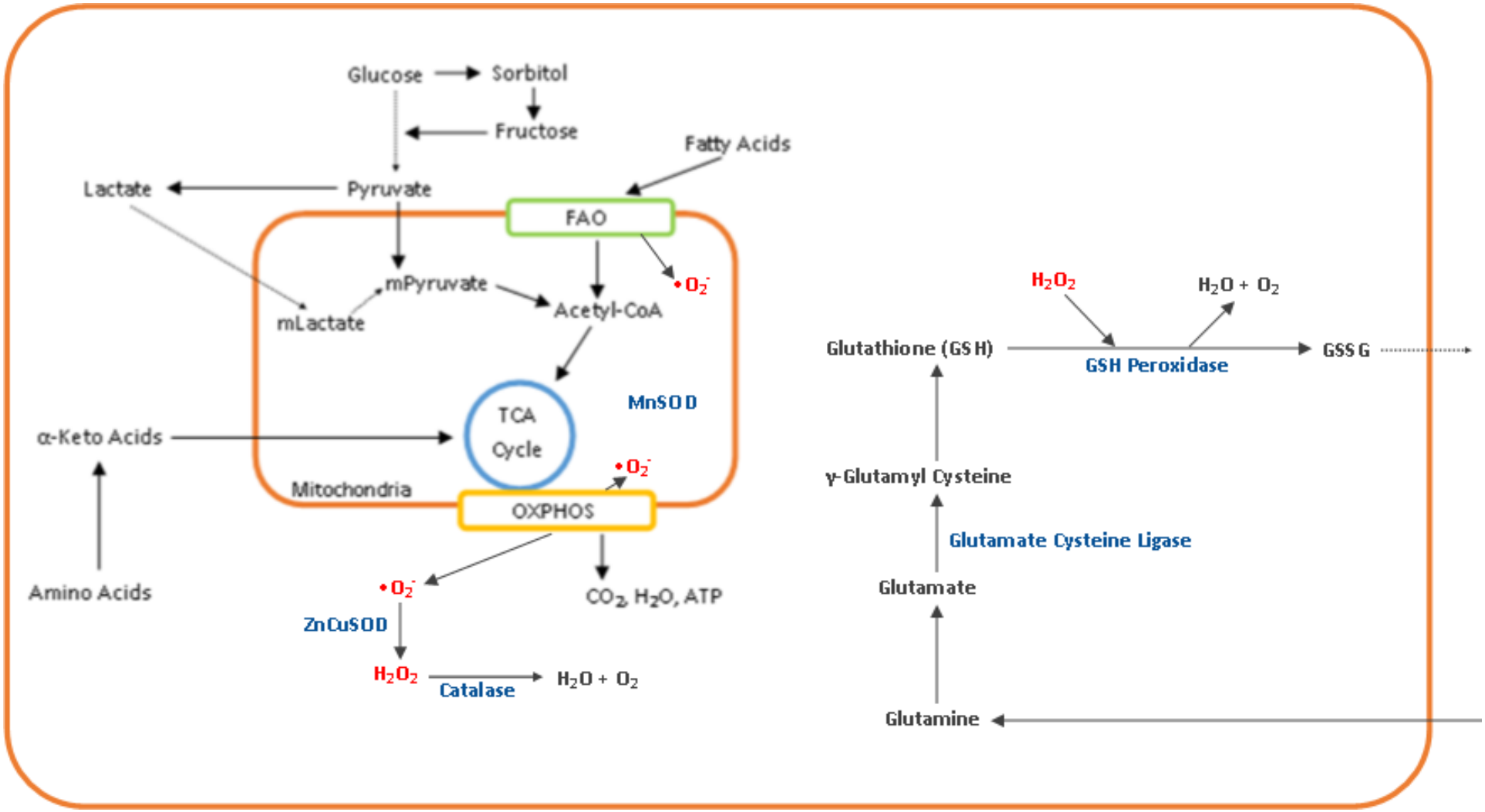

Figure 1.1. ROS production and defences in the mammalian cell. While there are a multitude of processes that produce ROS, the predominant one is Oxidative Phosphorylation in the Mitochondria. Glutathione is synthesised in the cell and is present in both the cytosol and the mitochondrial matric. Catalase catalyses the neutralisation of Hydrogen Peroxide to Water and Oxygen and is present in both the cytosol and mitochondrial matrix. Various Superoxide Dismutases (SODs) exist within the cell, including ZnCuSOD in the cytosol and MnSOD in the mitochondria.

As interest in ROS, oxidative stress and the use of anti-oxidants has gathered more pace in the medical industry, researchers in the biopharmaceuticals industry have started to investigate any link between oxidative stress and productivity in cultured cells. Work conducted by Underhill *et al* (2004) showed that Peroxiredoxin 1, an anti-oxidant enzyme, was significantly elevated in NS0 cells with increased Mab production, and high Mab producing CHO cells were shown to have an elevated glutathione pool (Chong *et al*, 2012), both studies therefore indicating a clear link between increased productivity and an increased resistance to ROS accumulation.

CHO cells and other mammalian manufacturing platforms have an elevated level of protein folding and secretion due to the recombinant gene product inserted into their genome. This increased activity within the Endoplasmic Reticulum inherently carries a risk of induction of the Unfolded Protein Response (UPR), and Du *et al* (2013) have shown that UPR incidence does increase in CHO cells producing recombinant proteins during fed-batch culture, a culture process representative of manufacturing. With an increase in UPR incidence comes an increase in ROS production (Santos *et al*, 2009), and studies have indicated that a reduction in ROS is linked to a reduction in ER stress, both through anti-oxidant supplementation (Malhotra et al, 2008) and through over-expression of cyclophilin B (Kim *et al*, 2008; Pybus *et al*, 2013).

In terms of metabolic flux, there have also been studies indicating a clear link between recombinant productivity and oxidative stress. The highest level of productivity in cultured cells is seen in the stationary phase of growth, and it has been shown that the expression of genes associated with the transport of amino acids related to the glutathione pathway such as alanine, cysteine, glycine and glutamate were found to be highly upregulated during the stationary phase of CHO cell culture (Kyriakopolous *et al*, 2013). The mitochondria, locus of energy generated through oxidative phosphorylation and main source of ROS within the cell, experiences increased flux during stationary phase and it has been postulated that glucose consumption in this stage of growth is used for NAD(P)H synthesis in order to counteract oxidative stress (Sengupta *et al*, 2010), and there is a peak in oxidative metabolism seen during high Mab synthesis (Templeton *et al*, 2013). Glutathione (GSH) flux is also increased during production, with a reduction in the redox pair GSH/GSSG seen during stationary phase (Selverasu *et al*, 2012) and late exponential phase (Templeton *et al*, 2013). It has been postulated that this reduction in GSH during production may be a cause of growth limitation and apoptosis in fed-batch production cultures (Selverasu *et al*, 2012).

1.3 Engineering Strategies Employed

Promising research into the use of anti-oxidants to combat oxidative stress coupled with this growing body of evidence indicating a link between redox capacity and productivity in CHO cell

factories informed the decision to further investigate this link in industrially relevant CHO cell lines and attempt to engineer oxidative stress resistance. Engineering strategies in such development can be limited only to cell line development, but is more effective when both the cell line and the process are developed with a view to improving the chosen characteristic (in this case, resistance to oxidative stress). It was hypothesized that ROS accumulation and the incidence of oxidative stress negatively impacts the growth and productivity characteristics of CHO cell factories. It was further hypothesized that it is possible to engineer oxidative stress resistance in CHO cell factories leading to improved growth and production phenotypes when transfected with a recombinant protein product. Two main engineering strategies were employed. First was a process engineering strategy targeting media formulation, attempting to design an anti-oxidant supplementation strategy to be utilized in a fed-batch culture process that will improve the characteristics of existing producer cell lines. The second was a directed evolution strategy, subjecting a host CHO cell line to steadily increasing levels of Hydrogen Peroxide in an attempt to generate cell lines with increased inherent oxidative stress resistance prior to transfection with recombinant genes.

1.4 Hypotheses

I. The accumulation of Reactive Oxygen Species and the subsequent Oxidative Stress has a negative impact on growth and productivity of industrially relevant CHO cell lines.
II. Oxidative Stress Resistance in CHO cell factories can be engineered through a Directed Evolution approach, employing a broad-acting pro-oxidant chemical (Hydrogen Peroxide) as the evolution pressure to generate Host Cell lines with an increased redox capacity.
III. Oxidative Stress Resistance in CHO cell culture processes can be engineered through basal growth and feeding media supplementation with anti-oxidant chemicals, leading to improved cellular performance in a fed-batch culture process.

Chapter 2:

Literature Review

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List of Abbreviations

CHO	Chinese Hamster Ovary	**α-T**	α-Tocopherol
ROS	Reactive Oxygen Species	**ALA**	α-Lipoic Acid
PTMs	Post Translational Modifications	**DHLA**	Dihydrolipoic Acid
BHK	Baby Hamster Kidney	**GSH**	Reduce Glutathione
HEK 293	Human Embryonic Kidney cell line 293	**GSSG**	Oxidised Glutathione
rP/rProtein	Recombinant Protein	**TNF/TNF-α**	Tumour Necrosis Factor
MAbs	Monoclonal Antibodies	**ARDS**	Adult Respiratory Distress Syndrome
q_P	Cell Specific Productivity	**BBB**	Blood Brain Barrier
COG	Cost of Goods	**MDA**	Malondialdehyde
CD	Chemically Defined	**HNA**	4-Hydroxynonenal
H_2O_2	Hydrogen Peroxide	**OTZ**	**L**-2-oxothiazohdine-4-carboxylate
O_2•	Superoxide	**BSO**	Buthionine Sulfoximine
•OH	Hydroxyl Radical	**5-OPase**	5-oxo-L-prolinase
RNS	Reactive Nitrogen Species	**MLN**	Melphalan
NO	Nitric oxide	**IC_{50}**	50% Inhibitory Concentration
NO_2	Nitrogen Dioxide	**AST**	Aspartate Transaminase
$ONOO^-$	Peroxynitrite	**NFκB**	Necrosis Factor κ-B
OXPHOS	Oxidative Phosphorylation	**TTP**	Triphenylphosphonium
FAO	Fatty Acid Oxidation	**NETs**	Neutrophil Extracellular Traps
SOD	Superoxide Dismutase	**mMP**	Mitochondrial Membrane Potential
ER	Endoplasmmic Reticulum	**PMA**	Phorbol 12-myristate 13-acetate
MFA	Metabolic Flux Analysis	**fMLP**	N-Formylmethionine-leucyl-phenylalanine
HP	High Producing	**SS**	Szeto-Schiller
LP	Low Producing	**IMM**	Inner Mitochondrial Membrane
BHA	Butylated Hydroxyanisole	**Dmt**	Dimethyltyrosine
NAC	N-Acetylcysteine	**EME**	Experimental Microbial Evolution

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2.1 Introduction

For the first time, this body of work investigates the nature of oxidative metabolism in industrially relevant Chinese Hamster Ovary (CHO) cells. Mechanistic underpinnings of the nature of oxidative phenotypes will be probed using pro- and anti-oxidant chemicals, and this knowledge will be used to engineer oxidative stress resistance in these cell lines.

All scientific endeavours must first start with a true understanding of the work that preceded them, and this can only be achieved through a thorough investigation of the relevant literature. Due to Intellectual property concerns, specific details regarding CHO use in the biopharmaceutical industry are often hard to come by. However, a cell type that has been used in scientific research since the 1960's inevitably leaves a paper trail regarding its culture processes, cellular behaviour and development requirements, and it is this vast body of work which informs any researcher embarking on CHO cell based research.

Oxidative stress and the accumulation of Reactive Oxygen Species (ROS) has received some interest in the biopharmaceutical field in the past 10 years, but the published literature is sparse and it is not currently an area affecting productivity that has been examined in any great depth. That being said, the topic ROS and the use of anti-oxidants to combat them has received renewed interest in the field of medical research; it is becoming increasingly clear that ROS accumulation plays a role in the vast majority of diseases, and anti-oxidants could be an inexpensive treatment option. There is, therefore, a wealth of knowledge available on the types of anti-oxidants available for use, their efficacy in different tissue types, and the mechanisms of their anti-oxidant properties. This is useful for our purposes, in that it provides a starting point for effective concentration ranges and ensures that different ROS production mechanisms are targeted in the study. The following chapter will examine the literature surrounding these topics, as well as investigating further the nature of evolution as an engineering strategy.

2.2 The Biopharmaceuticals Industry

2.2.1 The rise of CHO

The biopharmaceutical industry is a huge and steadily growing field, with global sales of biologics reported to be at US$140billion per annum in 2013 (Walsh, 2014), rising to US$163billion per annum in 2014, with an average annual growth rate of more than 8%, more than double that of the rest of the pharmaceutical industry (Otto *et al*, 2014). Often referred to as the "workhorse" of the biopharmaceutical industry, therapeutic recombinant protein production is currently dominated by Chinese Hamster Ovary (CHO) cell lines, with their use accounting for more than 70% of the market (Jayapal *et al*, 2007). The huge impact on efficiency and efficacy of recombinant therapeutic

proteins demonstrated by correct glycosylation and other post translational modifications (PTMs) for dominant rProteins such as Monoclonal Antibodies (MAbs) prompted a drive towards the use of mammalian over microbial cell cultures in industry, despite the higher financial implications. Even so, the choice of species is critical. Cell lines derived from human cells present concerns over viral transmission to patients in addition to their obvious ethical complications, and failings encountered during original trials before humanised MAbs (Purcell & Lockey, 2008) lead to the dismissal of murine cells as suitable candidates. With gene homologues for 99% of human glycosylating enzymes, it was found that CHO cells (as well as mouse myeloma NS20, baby hamster kidney (BHK) and human embryonic kidney (HEK 293) cells (Le *et al*, 2015)) did not generate the highly immunogenic Galα1,3-Galβ1,4-BlcNAc residues produced by mice (Butler, 2005; Xu *et al*, 2011), making them far more appropriate for recombinant protein production for use in human therapeutics. Over two decades of regulatory acceptance and optimisation of cell line development and production processes mark CHO as the "gold standard" industrial expression system. Optimisation strategies include genomic characterisation and titre optimisation, subsequent adaptation to suspension culture and serum-free media (Butler & Meneses-Acosta, 2012), as well as continued development and refinement for each new production requirement (such as improved secretory pathways, overcoming apoptosis and improvements in the glycosylation pathway (Le *et al*, 2015)).

Recombinant protein (rP) production, specifically production of monoclonal antibodies (MAbs), using mammalian platforms has increased 100 fold in the past two decades (Le *et al*, 2015) and achieved titres reaching up to 10 g/L (Kelley, 2009) for fed-batch culture systems. Such consistently high titres mean that production bottlenecks now usually arise at the cost of goods (COG) rather than ability to meet product demand (Le *et al*, 2015). However, whilst the processes for most therapeutic MAbs are established and highly productive, novel drug targets have shifted attention to so-called "difficult-to-express" rPs with a range of molecular moieties (O'Callaghan *et al*, 2015). Such biologics have persistently, and somewhat stubbornly low product titres (Pybus *et al*, 2013) despite usual approaches to both cell line and process development, suggesting a need for a more directed approach to developing either the cell line production platform, the process, or both (O'Callaghan *et al*, 2015).

2.2.2. Process Development: Cell Lines

Cell line development in an industrial setting is a time-consuming and costly process that is typically on the "critical path" to bringing a product to market. Therefore, for innovative biologics, developers will look to keep the cell line development time low (Le *et al*, 2015) whilst still identifying the best biomanufacturing compatible recombinant cell line, eliminating those cell lines which are deemed

"undesirable" (Porter *et al*, 2010). When lines are being developed, the cells are often sub-cultured every 3-4 days, which means they are always at a low density and have a plentiful nutrient supply, but also means that the culture never leaves the exponential stage of growth. Given that the optimal rP production stage is during the stationary phase of growth, an assumption has to be made that the cell line will behave during stationary phase as it did in the earlier stages of growth (an assumption which is often incorrect, see Stansfield *et al*, 2006 and McLeod *et al*, 2011). Further assumptions must also be made that upon progression from static plate based culture to bench top bioreactor, and subsequently, manufacturing scale bioreactor culture, the cells will exhibit the same biomanufacturing characteristics (Porter *et al*, 2010). Once discarded, "undesirable" cell lines are rarely analysed for performance, and there is therefore little data to show that such lines ever were truly unfit for purpose (Porter *et al* b, 2010). For this reason, it is preferable when assessing clonal quality during cell line development to ensure cells are cultured in processes as similar to the manufacturing platform as possible; fed-batch, and not batch trials should be conducted for cells that will be producing rP in fed-batch processes, for example (Le *et al*, 2015).

Ultimately, developers know that a high density of productive cell biomass will ensure the highest capacity for product formation, and maintenance of a high viable cell density for as long as possible will ensure a greater period of time for product formation (Stansfield *et al*, 2006). However, forcing cultured cells to exist in such a state can cause high levels of cellular stress, which can potentially have a damaging effect on product titre (McLeod *et al*, 2011) or even quality of that product (Palamores *et al*, 2004) , and so developers aim to strike a balance between increased growth and q_P, and decreased product quality.

2.2.3 Process Development: Media Formulation

As important as the decision on what cell line to use is the decision as to what media to grow it in. Media used for biotherapeutic production has long since been required in the large part to be animal component free, and manufacturers often choose to use commercially available chemically defined (CD) media with various commercially available feed strategies (Ling *et al*, 2015). Using serum free strategies has enabled manufacturers to ensure regulatory approval on this area of their production process, and chemically defined media also allows for a reduction in resources, and development and optimisation time lines, positively impacting COG (Ling *et al*, 2015).

In contrast to development of novel therapeutics, where there is a pressure to develop a process quickly enough to beat the competition and deliver the therapeutic to trials (Le *et al*, 2015), the production of biosimilars and the development of the biosimilar production process presents its own challenges. In advance of products coming "off-patent", companies will have more flexibility to

develop their cell line and production process in order to be competitive and can explore media additives and feeds more thoroughly, in order to reduce the COG burden (Le *et al*, 2015), while still aiming to be first to market. Chemically defined (CD) media is an industrial standard due to 1) animal free components largely being required for biopharmaceutical production, 2) commercially available CD media already having regulatory approval, thus reducing development time and cost and 3) quicker process development time when buying in commercial media rather than developing alternative proprietary versions. One obvious limitation with using commercial media, however, is that manufacturers have little to no information as to the exact formulation of the media that they are using, meaning cell line specific responses to specific additives cannot be readily observed. Chemically defined media will contain a range of components including salts, energy source, amino acids and trace elements in addition to a carbon source (Ling *et al*, 2015), as well as a range of metabolites to aid in stress reduction. However, the exact configurations remain closely guarded secrets on the part of the suppliers.

Due to these limitations, increasingly the goal for companies invested in biologics production is to develop proprietary media which can then be used to replace commercial media and supplement novel biologics production (Ling *et al*, 2015). Proprietary media in house would also enable security of supply for such companies, as they could choose to manufacture it at several sites.

2.3 Oxidative Stress and Antioxidants

2.3.1 Reactive Oxygen Species; disease and drug production

There is increasing evidence that many diseases afflicting the population, ranging from skin disorders (Bickers & Athar, 2006) to Hyperglycemia (Yu *et al*, 2005), Inflammatory Disease (Wiseman & Halliwell, 1996) and life threatening neurodegenerative conditions (Liu *et al*, 1999), have strong links to the accumulation of Reactive Oxygen Species (ROS; including Hydrogen Peroxide (H_2O_2), superoxide ($O_2^{\bullet}$) and Hydroxyl Radicals (•OH)) and, to a lesser extent, Reactive Nitrogen Species (RNS; including Nitric Oxide (NO), Nitrogen Dioxide (NO_2) and Peroxynitrite ($ONOO^-$)). ROS and RNS are generated during many biological processes. The greatest source is the mitochondrion (Balaban *et al*, 2005), producing ROS as a by-product of both Oxidative Phosphorylation (OXPHOS) (Muller *et al*, 2004; Murphy *et al*, 2009) and Fatty Acid Oxidation (FAO) (Seifert *et al*, 2010). The highly oxidised state of the mitochondrial microenvironment makes the valuable organelle the main target for oxidative damage. Mitochondrial DNA has been shown to suffer from oxidative damage, and it is theorised that lipid peroxidation caused by ROS leads to the release of caspase 6, rupturing of the mitochondria and eventual apoptosis (Ricci *et al*, 2001; Rahal *et al*, 2014). Other biologically

significant sources of ROS/RNS include metal catalysed reactions (Shahid, *et al*, 2014), cyotochrome p450 activity (O'Brien, 1991), and xanthine oxidase activity (Halliwell, 2011).

There are well documented defence mechanisms to prevent intracellular damage by ROS. These include the superoxide dismutases (SODs), which convert super oxide into hydrogen peroxide (Massaad *et al,* 2009; Hosoki *et al*, 2012), catalase for reduction of H_2O_2 (Wa *et al,* 2008; Glorieux *et al*, 2011) and glutathione peroxidase for the reduction of hydrogen peroxides and lipid peroxides (Lu, 2013). Glutathione acts as a scavenger of ROS (Anderson, 1998), and thus enzymes such as glutathione reductase can be considered indirectly to be antioxidant enzymes as they enable regeneration of the glutathione pool (Lu, 2013). Whilst cells have adapted to use ROS as signalling molecules to mitigate the damage they can cause (Thannickal *et al*, 2000), oxidative stress is the term given to the phenomenon when an imbalance in the redox status of the cell occurs due to cellular defence mechanisms becoming overwhelmed and ROS are allowed to accumulate to a toxic level. Oxidative stress leads to large-scale damage in DNA, proteins and lipids and can eventually lead to apoptosis, necrosis and tissue damage (Sies, 2015). This is due to the effects of ROS on cellular molecules, such as H_2O_2 causing lipid peroxidation (Ayala *et al*, 2014) and toxic aldehydes produced from amino acid oxidation (Dorźdz *et al*, 1988).

Since ROS are so critical in so many diseases, it stands to reason that such molecules, and the phenomenon of oxidative stress would have a detrimental impact on the behaviour of cell lines used for biologics production. The increased metabolic burden of producing therapeutic recombinant proteins means commercial cell lines are producing more ROS due to the increased need for energy (ROS production from OXPHOS) and protein folding and secretion (ROS production in the ER, Malhotra *et al*, 2007), and are thus more susceptible to oxidative stress. Keightley *et al* (2004) showed that an increased resistance to ROS leads to increased productivity in cultured cells, and were able to engineer fibroblast cell lines with higher catalase activity and stronger resistance to oxidative injury that also showed increased rProtein production. Shortly after this work, in 2008, Malhotra *et al* showed that introduction of anti-oxidant compounds to the culture process reduced ER-stress, oxidative damage and improved protein secretion in CHO-H9 cells. Further, Metabolic Flux Analysis (MFA) of High Producing (HP) and Low Producing (LP) CHO cells in culture showed that glutathione metabolism shifted towards an outward flux of oxidised glutathione from the cells during stationary phase, indicating a decreased reducing capacity and increased likelihood of oxidative stress in cells during their peak production period (Selverasu *et al*, 2012). In order to mitigate the detrimental impact on production ROS may have in cultured cell lines, many commercial CD media are likely to contain quantities of antioxidant chemicals. Despite some media optimisation work conducted during process development, manufacturers are unaware of which

antioxidants have been included in their chosen commercially available media, in what concentrations, and there is no published data to show that they work.

2.3.2 Antioxidant chemicals

Broadly speaking, antioxidants can be separated into categories based on their mode of action. Scavenging compounds such as ascorbic acid and uric acid have long been known for their antioxidant properties. Such molecules act by reducing excess ROS, and are largely considered to be specific for certain oxidative species (Yen *et al,* 2002). Other antioxidant compounds such as Selenium (Leist *et al*, 1996) and Butylated Hydroxyanisole (BHA) (Martin *et al*, 2014) act in a more indirect manner, enhancing the activity of the enzymes such as caspases and superoxide dismutases (SODs), whilst L-2-oxothiazolidine-4-carboxylate (Procysteine) and N-Acetylcysteine (NAC) restore the cellular glutathione pool (Bernard *et al*, 1997; Dizdar *et al, 2000*). In addition to naturally occurring antioxidants, a series of mitochondrially targeted molecules have been described, and often tend to be much more potent than the untargeted counterparts. The growing wealth of knowledge regarding the responsibility of ROS in a broad range of diseases means that antioxidants are seen as promising candidates for therapeutics. However, despite this knowledge and the many successful and promising studies *in vitro* a lot of the progressions into animal and clinical studies have been disappointing. The so-called "antioxidant paradox" described by Halliwell in 2000 discusses the disappointing results in clinical trials utilising Vitamin E to prevent cardiac disease. Patients did experience the lipid peroxidation in target cells as predicted, but this did not prevent disease. The paradox was that while *in vitro* studies can be promising, individuals within a population may be pre-disposed to responding to anti-oxidant treatment differently due to their own genetics or metabolism, and that targeting the disease is not the only factor when using anti-oxidants as disease treatment (Halliwell, 2000). It has become clear that antioxidants will only be successful as treatment options if this paradox can be addressed.

2.3.2.1 Scavenging Chemicals

There are numerous compounds that can act as so called ROS scavengers, mopping up reactive species within the cell and its organelles. Discovered in 1922 (Evans & Bishop, 1922), Vitamin E has had a chequered past with regards to its antioxidant activity. Comprised of 4 tocopherols and 4 tocotrienols (α-, β-, γ- and δ- for both groups), it appears that *in vivo* α-tocopherol (α-T) appears to be the only one in the Vitamin E family that is taken up in significant amounts (Cervinkova *et al*, 2016) and it is often this (occasionally along with α-tocotrienol) that is used in antioxidant studies.

Defined as a radical chain-breaker, α-T operates in a lipid environment and is often described as protecting against lipid peroxidation (Cervinkova *et al*, 2016) through reduction of

peroxyl radicals (Equation 2.1). Evidence from 1981 indicated that all tocopherols act in a chain-breaking capacity and that α-T was able to perform better than any synthetic antioxidant of the time, having a much larger rate constant for H^+ transfer to peroxyl than any other (Burton & Ingold, 1981). Such studies indicated that Vitamin E therefore was able to act in an antioxidant manner in lipid and phospholipid suspensions (Burton & Ingold, 1981) and strengthened the argument for the use of α-T in *in vitro* investigations. Studies in liver microsomes and liposomes indicated that both α-tocopherol and α-tocotrienol displayed antioxidant activity, as defined by a decrease in lipid peroxidation, and through a proposed mechanism of interaction with lipid peroxyl radicals (Traber & Atkinson, 2007.

$$ROO\cdot + Toc\text{-}OH \rightarrow ROOH + Toc\text{-}O\cdot \qquad \textbf{Equation 2.1}$$

Whilst unable to regenerate itself, there is evidence to suggest that when used synergistically, Ascorbate (Vitamin C) has the ability to regenerate oxidised α-T and thus make it available for further protective behaviour (McCay, 1985). The majority of studies into this relationship, however, are *in vitro* and further probing of the observed chemical relationship and its relevance in deficiency related disease are needed to elucidate its impact *in vivo.* α-Tocopherol continues to be used in antioxidant studies, with papers published within the last 6 months utilising this phenolic compound (Cervinkova *et al*, 2016; Hosain *et al*, 2016; Shin *et al*, 2016, to name just a few). However, questions about its antioxidant properties were raised as early as the 1950s, and Azzi's review in 2007 indicates that many researchers still disagree that Vitamin E's beneficial properties stem from an intrinsic antioxidant property.

Ascorbic Acid is an antioxidant compound with iron chelating activity that has been included as a supplement in animal feed for decades in order to improve energy levels and growth of livestock (Lohakare *et al*, 2005), and is listed on WebMD as being an appropriate dietary supplement to improve immune system deficiencies, cardiovascular disease and eye disease. However, despite its known antioxidant activity *in vitro* (Yen *et al*, 2002), trials investigating its worth in treatment of diseases related to oxidative stress have largely been unsuccessful. This is likely to do with its dual activities as a scavenging antioxidant and as an iron chelator, activities which are more prominent at different active concentrations. Whilst most antioxidant compounds only become toxic when used in very high concentrations, Ascorbic Acid has a specific active concentration range which means too low a dose will also result in toxicity, specifically through DNA damage (Yen *et al*, 2002), and at too high a dose the chelating activity of the molecule can result in it promoting production of $OH^{\bullet}$ (Yen *et al*, 2002). Thus, an effective concentration for antioxidant activity of the compound can also, *in*

vivo, cause partial chelation of iron and so production of ROS, and its beneficial effects are reduced or eliminated completely.

The redox couple α-lipoic acid/dihydrolipoic acid (ALA/DHLA) was first identified in the early 1950s (Reed, 1957), and the synergistic antioxidant properties of this couple have led to the nickname of the "universal antioxidant". In fact, with a redox potential of -0.32*V*, this antioxidant couple is actually more potent than the GSH/GSSG couple (-0.24*V*) (Moini *et al*, 2002). The ability to be readily absorbed into the cell, to quench ROS in both the aqueous and lipid phase, to chelate metals and to regenerate other antioxidants makes this couple an extremely exciting prospect for any researcher looking to investigate the oxidative state of their system.

α-Lipoic acid is able to scavenge hydroxyl radical, hypochlorous acid and singlet oxygen at an *in vitro* concentration range of 0.05-0.1mM (Moini *et al*, 2002) but is not able to scavenge peroxyl radical, whilst *in vitro* experimentation indicates DHLA at concentrations of 0.01-0.5mM is able to scavenge peroxyl radical, superoxide radicals in addition to hydroxyl radicals and hypochlorous acid (Packer *et al*, 1995). In addition to the potent antioxidant activity seen *in vitro*, ALA is one of the few anti-oxidant compounds that has been positively used *in vivo* to combat oxidative stress-related diseases. Promising treatment applications include for Diabetes (Packer *et al*, 2001), Hypertension (Vasdev *et al*, 2000), AIDS (Patrick *et al*, 2000) and Hepatic Disorders (Bustamante *et al*, 1998).

Butylated Hydroxyanisole (BHA) is a phenolic compound traditionally used as a preservative in tinned or canned foods (Yu *et al*, 2000), and was recognised as having antioxidant scavenging activity at a concentration range 50-100μM when added to culture medium in both primary and established cell lines (Vercammen *et al*, 1998; Moon & Park, 2011). In addition to this, BHA has been shown to prevent oxidative-stress related apoptosis in a range of human cells including neurons (Ratan *et al*, 1994), monocytes and macrophages (Jin *et al*, 2012; Zhang *et al*, 2013), and inhibited leukemia (Okubo *et al*, 2004) and HeLa cancer cells (Moon & Park, 2011). As with all antioxidant compounds, however, BHA acts as somewhat of a double-edged sword, and its beneficial effects appear to be largely tissue dependent. For example, in intact rat hepatocytes, BHA was shown to destroy the Mitochondrial Membrane Potential (MMP; ΔMψ) leading to calcium ion loss, mitochondrial swelling and decrease in ATP levels which are ultimately cytotoxic (Yu *et al*, 2000); in contrast, Tumour Necrosis Factor (TNF)-induced apoptosis is inhibited by the same compound in mouse fibrosarcoma cells (Vercammen *et al*, 1998). The antioxidant activity of BHA is dependent on the type of stress induced, with Verhaegen *et al* (1995) demonstrating that BHA treatment was able to reduce UV- and H_2O_2-induced cell death in HL-60 cells lasting up to 6 hours after irradiation, but that there was minimal effect on drug-induced apoptosis.

2.3.2.2 Glutathione Synthesis and Glutathione Pool Support

One of the cell's major endogenous defences against oxidative stress is the glutathione pool. Glutathione peroxidase (GSH Peroxidase) catalyses the reaction between GSH and ROS, thus neutralising the ROS and leaving oxidised glutathione (GSSG), which is then recycled back to GSH by GSSG reductase, making GSH available once more to scavenge ROS (Lu, 2009; Dickinson & Forman, 2002; Forman *et al*, 2008). GSH can be directly supplied to support the protective redox pool, but it must be metabolised extracellularly before being taken up into the cell, and so intracellular GSH levels often do not differ greatly from unsupplemented cells when using treatment of this kind (Lu, 2009). It is therefore a better solution to improve glutathione synthesis within the cell, the rate limiting step of which is incorporation of **L**-cysteine by the enzyme glutamate-cysteine ligase (formerly γ Glutamyl-cysteine Synthetase) (Cai *et al*, 1997) (Figure 2.1). Direct supplementation of cysteine to support GSH synthesis, however, is not appropriate due to its cytotoxicity, leading to supplementation with cysteine deliverers such as *N*-Acetylcysteine (NAC) and **L**-2-oxothiazohdine-4-carboxylate (Procysteine) (Dizdar *et al,* 2000).

A thiol-containing derivative of cysteine, *N*-Acetylcysteine has been used widely therapeutically since the 1950's, being used as a treatment for certain types of lung disease, paracetamol intoxication, and attempts have been made to use it to treat pulmonary conditions including Adult Respiratory Distress Syndrome (ARDS) (Bernard *et al*, 1997). More recently, it has been used as a glutathione precursor for research applications in studies probing apoptosis regulation and gene transcription (Dizdar *et al*, 1999), as well as being investigated in relation to inflammatory and immunological disorders (Kim *et al*, 2014; Akawara & Ito, 2007). NAC acts as an effective scavenger of hydroxyl radicals, superoxide and hydrogen peroxide (Dizdar *et al*, 1999). However, in addition to this role, NAC can be de-acetylated by N-acetylases to form **L**-cysteine as a precursor for GSH synthesis (Dizdar *et al*, 1999). This duality, as well as the drug's lack of accumulation in the body and its suggested ability to cross the Blood Brain Barrier (BBB) has led to calls for clinical trials in the use of NAC to treat disorders directly associated with oxidative stress (Kim *et al*, 2014).

In a 1996 study, Ercal et al showed that 1mM NAC conferred protection from Lead (Pb) damage up to 1.0 mg/mL in Chinese Hamster Ovary (CHO) cell cultures. Their work demonstrated that NAC treatment was able to restore GSH concentrations to control levels, as well as lowering GSSG to control concentrations. NAC was also shown to lower Malondialdehyde (MDA) levels – an indicator of lipid peroxidation caused by ROS – as well as to lead to a decreased activity in antioxidant enzyme catalase, in comparison to cultures treated with Pb only. An *in vivo* study by

Dizdar *et al* (1999) compared the efficacies of NAC (2 mmol/kg) and Procysteine (OTZ) (2 mmol/kg) at protecting mice from oxidative damage caused by Buthionine Sulfoxamine (BSO) treatment.

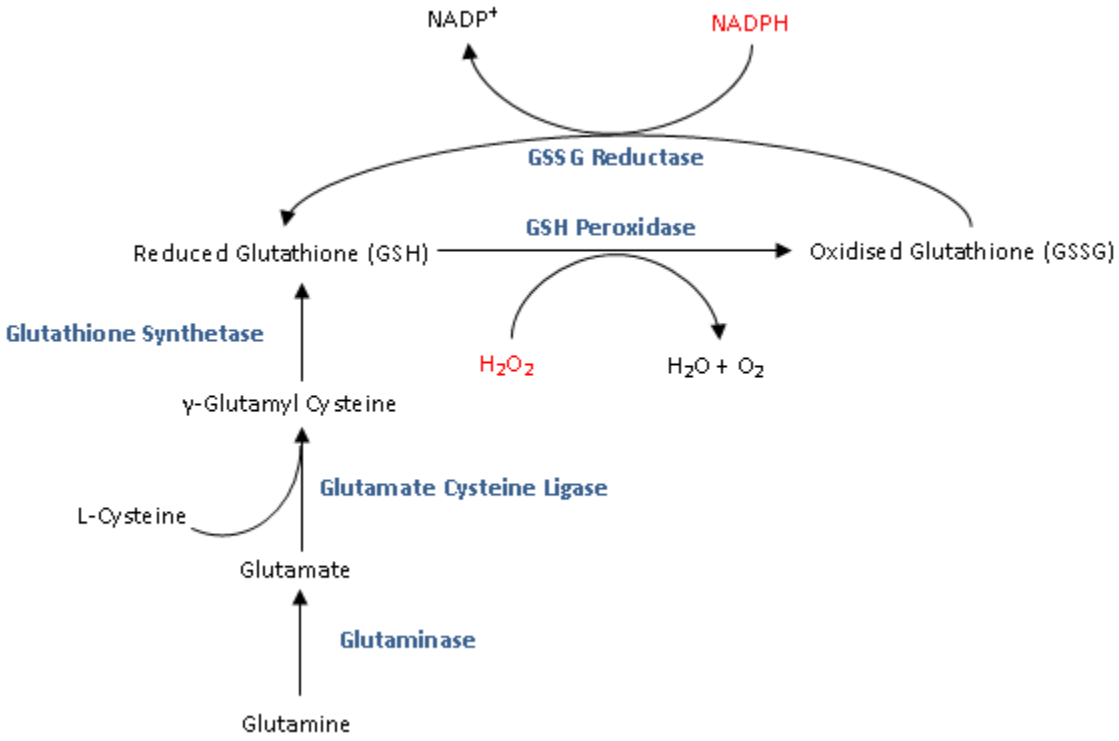

Figure 2.1. The redox recycling of glutathione.

An inhibitor of glutamate-cysteine ligase, BSO reduces the synthesis of GSH, thus reducing the cell's capacity to protect from oxidative damage. Dizdar *et al* (1999) found an increase in interstitial cysteine concentrations following NAC administration post cessation of BSO treatment, reinforcing NAC's role of cysteine deliverer.

In a review published in 2007, Arakawa and Ito detailed the current pharmacological knowledge regarding NAC and its role in neurodegenerative diseases, and highlighted the potential for clinical trials of NAC as a treatment option. A combinatorial treatment of low levels of NAC followed by treatment with Ebselen, a lipid-soluble seleno-organic compound, led to decreased HNE-induced death and increased GSH production in neurons. The authors present evidence suggesting the potential for NAC treatment in cases of Unverricht-Lundborg myoclonus epilepsy, Alzheimer's Disease, Parkinson's Disease, Tardive Diskinesia and Down's Syndrome, but highlight the lack of extensive clinical trials despite promising initial studies and anecdotal accounts of success (Arakawa *et al,* 2007).

L-2-oxothiazohdine-4-carboxylate (OTZ, Procysteine) is an analogue of 5-oxo-**L**-proline, a contributor to the initial stage of GSH synthesis, and is used as a cysteine deliverer in studies of oxidative stress (Dizdar *et al*, 1999; Iimuro *et al*, 1999). 5-oxo-L-prolinase (5-OPase) is required to convert OTZ to L-cysteine, and it has been observed that tumour cells have a lower concentration of this enzyme than normal tissue (Chen & Batist, 1998). It has been shown that application of OTZ in rats saw an increase in cellular GSH content of normal tissue, but a decrease in tumour cells (Otis & Guidot, 2009). Chen *et al* (1998) showed that the anticancer drug melphalan (MLN) had increased cytotoxicity when used in conjunction with OTZ, indicating OTZ's role as a chemosensitising agent. This sensitising effect was further confirmed by the same group, when they treated tumour cells with a combination of 10mM OTZ and MLN at its IC_{50} concentration.

Procysteine has been shown to protect liver cells against oxidative damage due to alcohol consumption in several studies. Male Wister rats fed on an ethanol containing diet were treated with 500 mg/kg/day dietary OTZ; rats without OTZ displayed increased serum aspartate transaminase (AST), necrosis and inflammation along with a 2-3 fold increase in hepatic tumour necrosis factor α (TNF-α), mRNA and nuclear transcription factor nuclear factor κB (NFκB) (Iimuro *et al*, 1999). These effects were blocked or dampened by OTZ. Plantaris muscle atrophy in alcohol-fed Sprague-Dawley rats was attenuated following OTZ treatment (0.35% w/v) (Otis & Guidot, 2009). In addition, glutathione levels were restored and activity of antioxidant enzymes was increased, along with an increase in gene expression of anabolic factors. The authors of this latter study noted that OTZ did not completely block alcohol induced oxidative stress, but minimised the impact by increasing production of anabolic pathway components (Otis & Guidot, 2009).

With similar modes of action, it can be difficult to decide whether to use NAC or Procysteine as a cysteine deliverer, and the evidence would suggest that consideration of tissue type is important in this decision. Studies in liver cells suggest a preference for OTZ (del Olmo *et al*, 2000), and investigations into the efficacy of treating ARDS indicated OTZ to be slightly more potent (Bernard *et al*, 1997). However, Dizdar *et al* (1999) found NAC to be a more effective cysteine deliverer when treating melanoma cells.

2.3.2.3 Mitochondrially Targeted Antioxidants

The greatest source of ROS in the cell is the mitochondrion, producing ROS as a by-product of both Oxidative Phosphorylation (OXPHOS) (Poyton *et al*, 2009) and Fatty Acid Oxidation (FAO) (Seifert *et al*, 2010). The highly oxidised state of the mitochondrial microenvironment makes the valuable organelle the main target for oxidative damage. Mitochondrial DNA has been shown to suffer from oxidative damage, and it is theorised that lipid peroxidation caused by ROS leads to the release of caspase 6, rupturing of the mitochondria and eventual apoptosis (Lowes *et al*, 2008).

Despite promising *in vitro* and *in vivo* studies, many clinical trials with antioxidants have yielded little success (Lowes *et al*, 2008). It has been suggested that one of the reasons these compounds are ineffective *in vivo* is the inability to localise to the sites of ROS production, thus diluting their reducing effects (Lowes *et al*, 2008). On the back of this theory, a number of antioxidant compounds targeted to the mitochondrion have been discovered or synthesised, with their potency appearing to be orders of magnitude higher than the untargeted alternatives.

The first of the mitochondrially targeted compounds to be deliberately synthesised, MitoQ comprises a lipophilic triphenylphosphonium (TTP) cation attached to the antioxidant ubiquinone moiety of the endogenous coenzyme Q10 (Kelso *et al*, 2001). The TTP cation allows MitoQ to easily be taken up through the cell and mitochondrial membranes, and the high potential of the energized inner mitochondrial membrane causes it to accumulate a hundred fold more in the mitochondrion than the rest of the cell (Kelso *et al*, 2001). Once inside the mitochondria, the MitoQ adsorbs to the matrix face of the inner-membrane (Rao *et al*, 2010).

MitoQ synthesis was first reported in 2001 by Kelso *et al*, and the compound was tested for efficacy in both isolated mitochondria and intact cells. In isolated mitochondria, MitoQ was ineffective up to 10 μM whilst at 25 μM and above membrane potential was reduced. In intact human 143B cells, 10 μM MitoQ did not affect cell viability and 25-50 μM lead to cell death (Kelso *et al*, 2001). Tests in Jurkat cells with 5 μM MitoQ showed the novel compound was able to prevent H_2O_2 induced apoptosis only, suggesting its ability to mediate the negative impacts of oxidative stress upon the cell (Kelso *et al*, 2002). Several animal models of disease have been tested with MitoQ; rats fed with MitoQ showed decreased heart dysfunction and mitochondrial damage on ischemia-reperfusion (Lowes *et al*, 2008); rat models of oxidative stress also saw increased protection of endothelial cell function and mitochondrial enzymes (Lowes *et al*, 2008). In addition to the animal models, MitoQ is also now being developed as a human pharmaceutical, with phase II trials showing protection against liver damage in hepatitis C patients (Tauskela, 2007).

The recyclable nature of MitoQ that Kelso *et al* (2001) described has not proven to be as beneficial as they hoped, however. Rather than providing continual antioxidant defence, it has been shown that continual exposure to MitoQ leads to an increase in ROS production in both isolated mitochondria and intact cells (Lowes *et al*, 2008). This is down to an increase in OXPHOS upon administration of MitoQ, and the subsequent proton leak destroying the mMP (Rao *et al*, 2010). Dose is therefore an important consideration in studies, to ensure that the antioxidant benefits of the ubiquinone moiety are not undone by the pro-oxidant effects of its accumulation.

Similar to MitoQ, MitoTEMPO is a superoxide dismutase mimetic bound to a lipophilic TTP in order to target the anti-oxidant chemical to the mitochondria. Developed by Dikalova *et al* (2010),

they were able to show a 3-fold higher concentration of MitoTEMPO in the cytoplasm compared to the extracellular media, and substantial accumulation in the mitochondria up to 15 µM after incubation for an hour. Trnka *et al* (2009) showed that lipid peroxidation in bovine heart mitochondrial membranes was reduced when using 1 µM concentrations of MitoTEMPOL, and that in *in vitro* systems, MitoTEMPOL-H was just as effective at decreasing lipid peroxidation as the other popular mitochondrially targeted anti-oxidant compound, MitoQH_2. In a study investigating programmed cell death in neutrophils that have released neutrophil extracellular traps (NETs), Vorobjeva and Pinegin (2015) were able to show that the non mitochondrially targeted form of this compound, TEMPOL, was able to suppress phorbol 12-myristate 13-acetate (PMA)-induced ROS release and inhibit fMLP-induced neutrophil degranulation at concentrations of 0.1-5 mM, indicating that targeting to the mitochondria allows for a much lower active concentration.

Szeto-Schiller (SS) peptides are a series of small, cell-permeable antioxidant peptides that accumulate in the inner mitochondrial membrane (IMM). First reported in 2004 by Zhao *et al* after discovery during an opioid receptor study, the peptides feature alternating aromatic residues and basic amino acids, and their antioxidant activity is attributed to the dimethyltyrosine (Dmt) residue (Szeto, 2006), a tyrosine residue with enhanced ROS scavenging activity due to methylation of the phenolic ring. SS peptides did not exhibit toxicity at concentrations up to 100 µM (Cao *et al*, 2012), in contrast to other mitochondrially targeted antioxidants such as MitoQ, and this is down to them not having a lipophilic cation moiety and thus altering the mitochondrial membrane potential upon accumulation (Cao *et al*, 2012). Studies have shown that SS peptides can scavenge hydrogen peroxide (H_2O_2) and peroxynitrite, reduce mitochondrial ROS production, and prevent mitochondrial swelling and lysis by protecting the membrane from lipid peroxidation (Li *et al*, 2011; Cao *et al*, 2012; Szeto, 2006).

The low toxicity and high solubility of SS peptides has made them highly popular in redox research, with SS31 appearing to be the most potent and therefore highly favoured. Both Cao *et al* (2010) and Li *et al* (2011) used SS31 to study the impact of reducing mitochondrial ROS in models of hyperglycaemia, both showing significant mMP protection at concentrations as low as 100 nM. Animal models tested with SS31 have also shown high efficacy for burn-induced insulin resistance (Carter *et al*, 2011), renal fibrosis (Mizuguchi *et al*, 2008), ischemic brain injury (Cho *et al,* 2007) and neurodegeneration (Moreira *et al*, 2010). Of the mitochondrially targeted antioxidants available, the SS peptides appear to be the most promising as druggable compounds for treatment of the plethora of diseases associated with ROS accumulation and oxidative stress.

2.3.2.4 Anti-oxidants in CHO Cell Culture

Much of the research investigating the efficacy of anti-oxidant chemicals, as can be seen here, is centered around medical applications. While much less frequent, there is however research into how the use of anti-oxidants can affect the performance of CHO cells in culture. Table 2.1 gives a brief overview of this work, detailing the impact these anti-oxidant chemicals had on the cell lines trialled.

Table 2.1. Anti-oxidant Use in CHO Cell Culture. A brief overview of the use of anti-oxidants in CHO cell research, with the observations of the researchers and appropriate references. Abbreviations: MMP: Mitochondrial Membrane Potential; FBS: Foetal Bovine Serum; ROS: Reactive Oxygen Species; ER: Endoplasmic Reticulum; IFN-β: Interferon-β-1a; Pb: Lead; GSH: Glutathione.

Name	Mode of Action	Concentration Used	Observations	References
Ascorbic Acid	ROS Scavenger	10 mM	Increased viability in low-FBS media; Increased incidence of cells with high MMP	Yun et al, 2001
Butylated Hydroxyanisole	ROS Scavenger	10 µM	Reduced apoptosis in sodium butyrate treated cells; Reduction in oxidative stress in sodium butyrate treated cells; Increased rProtein secretion; Decreased incidence of ER stress in sodium butyrate treated cells	Malhotra et al, 2008
Glutathione	Glutathione Pool Support; ROS Scavenger	10 mM	Increased viability in low-FBS and FBS-free media; Increased incidence of cells with high MMP; Reduced intracellular ROS levels	Yun et al, 2001; Yun et al, 2002
N-Acetylcysteine	Glutathione Pool Support	1 – 10 mM	Increased rProtein titre; Extended culture period timeline; Improved rProtein quality (terminal sialylation of recombinant IFN-β)	Oh et al, 2005
Taurine	Reduction of superoxide generation through regulation of mitochondrial protein synthesis	10 mM	Decreased lipid peroxidation in Pb treated cells; Increased GSH levels in Pb treated cells; Decreased Catalase and glucose-6-phosphate dehydrogenase activity in Pb treated cells	Gürer et al, 2001; Jong et al, 2012

2.4 Directed Evolution in Cell Line Engineering

The nature of CHO-based biopharmaceutical production culture methodology means much of their behaviour draws a parallel with more traditional suspension cultures of microbes. Cells maintained in routine subculture are continually subjected to what are known as population bottlenecks due to standard splitting techniques. This term, "population bottleneck", is used to describe the process of taking a random section of a culture of micro-organisms (or in the case of mammalian biopharmaceutical production, a culture of single cells) at the end of a specified growth period and supplanting it into fresh growth media at a defined dilution ratio (Wahl *et al*, 2002). It has been

shown that repeated population bottlenecks, especially when they continually occur during the exponential phase of growth as in routine subculture, can encourage evolution of cells within the population (Wahl *et al*, 2002). Although these mutations may be beneficial, it is not guaranteed that beneficial mutations will be retained when subjected to repeated bottlenecks, and detrimental mutations are just as likely to be retained when there are no other external stressors or selection pressures (Wahl & Krakauer, 2000).

Genetic drift due to repeated bottlenecks is, as discussed, random and unpredictable. Uncontrolled genetic variation is not usually desirable in experimental or industrial contexts, and so in order to avoid this genetic drift impacting on experimental outcomes, it is common practice to restrict the number of host cell subcultures during routine maintenance or cell line development to less than 20 (roughly 60-70 population doublings). Recombinant cell lines will typically experience >590 population doublings post cryo-revival, but stability of cell lines at this point tends to be measured in terms of cell line behaviour (i.e. growth and productivity levels) along with product quality, rather than genetic stability. However, it is not always disadvantageous to encourage or even direct genetic drift. Such direction can be achieved by introducing a selection pressure and maintaining it during repeated population bottlenecks, thus encouraging desired mutations to be maintained (Wahl & Gerrish, 2001). This directed evolution method represents one of the two main methods of introducing new, desirable phenotypes, with the other being rational design. The benefit of directed evolution over rational design when attempting to engineer cell lines is that it requires little specific knowledge on the mechanisms of the target process to be engineered. Everything from enzymatic pathways, signalling networks and cascades, to the production of specific proteins can all be targeted with application of the correct selection pressure, without needing to account for potential signalling redundancy which would possibly prevent engineering through rational design of enzymes from altering cellular phenotype (Adams & Rosenzweig, 2014).

2.4.1. The History of Experimental Microbial Evolution

Experimental Microbial Evolution (EME) as it is now know can find its origins in the early 1940s, with the first description of the design, construction and use of a chemostat by Jacques Monod (referred to by him as a "Bactogen" (Monod, 1942). Whilst he was interested in using such a device to investigate growth rates under limiting nutrient concentration, he recognised its use in the interrogation of the nature of evolution and "mutability" (Adams & Rosenzweig, 2014). It is then possible to isolate three papers published around the same time in the 1950's that describe the

three recognised methods of continuous or extended culture used to analyse genetic changes; Novak and Szilard reported the use of the chemostat (1950); Bryson and Szybalski described the use of the turbidostat (1953); Schneider and Ryan described using serial dilution (Atwood *et al*, 1951).

Over the next 20 years, the literature documents use of EME as a tool to gain insights into the enzymatic and genetic structures of unicellular organisms (Adams & Rosenzweig, 2014), but this was work done by biochemists and microbiologists; evolutionary scientists did not at first recognise EME as the powerful tool it could be in modelling evolutionary processes in the macro scale (van de Ende, 1973; Paynter & Bungay, 1969). The primary goal of the work done in these junior years of EME was an increased understanding of the biochemistry and physiology of their target organisms, however hindsight indicates they had much wider implications for advancing the understanding of the genetic basis of evolutionary change (Dykhuizen, 1978; Levin & Lenski, 1983). Throughout the 1980's, the power of EME to model evolution on the macro scale began to be truly recognised. The increased resolution of data available in terms of model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* due to the far more powerful genetic mapping tools available through EME led to an understanding that monocellular asexual organisms were able to evolve under nutrient limited conditions in as little as 100 generations (Helling *et al*, 1987). Thus evidence was emerging that contradicted the two basic tenets of evolutionary theory: both the classical model of evolution of asexual organisms (Muller, 1932), and the competitive exclusion theory (Gause, 1934) were challenged thanks to data generated using Experimental Microbial Evolution, an observation that was interesting to traditional evolutionary scientists and thus sparked more interest in this emerging field (Adams & Rosenzweig, 2014).

The advent of Whole Genome Sequencing, though largely driven by the Human Genome Project and the desire to advance medical science, was never the less the final leap needed to advance EME to the status that it holds today. Due to their small relative size and simplicity, the first genomes sequenced were microbial, including key model organisms such as bacteriophage lambda (Sanger *et al*, 1982) and *E. coli* (Blattner *et al*, 1997), and with *S. cerevisiae* being the first fully sequenced eukaryote, biochemical and microbiological researchers were able to further develop techniques such as micro-arrays that enabled them to start mapping and collating the relative genomes (Lashkari *et al*, 1997), transcriptomes and proteomes (Schwikowski *et al,* 2000; Ferea *et al*, 1999) and metabolomes (Philippe *et al*, 2007) of these simple, but important organisms. Thus, the predictions of Lewontin from the 1970's about the need for understanding of the significance of genetic variance in order for evolutionary biology is now starting to be realised.

2.4.2. Harnessing the power of Experimental Evolution

Whilst the power of microbial evolution is now being fully recognised in the academic fields associated with both evolution and microbiology, when justifying experimental evolution as a method to engineer novel cell lines, it is necessary to elucidate whether such a long term engineering strategy is likely to produce desirable outcomes. Lindi M. Wahl has produced many articles discussing the mathematical nature of experimental vs natural evolution, and attempting to develop mathematical models to predict the occurrence, retention and fixation of mutations, and more specifically beneficial mutations.

The experimental life cycle of any cells in culture features a specific aspect critical in the evaluation of an evolution experiment: population bottlenecks (Wahl & Gerrish, 2001). Each time cells are sub-cultured they experience a bottleneck, whereby the rate of their growth is stalled and they must adapt to their new environment. It is important to researchers to ensure that survival is not merely an adaptation by the cells to survive these bottlenecks, and the process by which the dilution occurs is deliberately as random as possible to ensure a random selection of the cell population. Most mutations in a population will occur, by a matter of probability, when the population is highest (Wahl *et al*, 2002) – thus they will occur just before the point of dilution. Thus, frequent reduction in population size is likely to have a detrimental effect on the likelihood of beneficial mutations arising and then fixing within the population (Wahl *et al*, 2002). On the other hand, maintaining a population in exponential growth phase increases the likelihood that mutations will arise due to the high rate of cellular division (Wahl *et al*, 2002). Maintaining cells in exponential growth phase while establishing the optimal dilution ratio (i.e. the optimal volume to take from the old population and inoculate into new media) will increase the likelihood of mutations occurring, and increase the probability of beneficial mutations fixing into the population (Gifford *et al*, 2013). Wahl *et al* established that in standard conditions, beneficial mutations are no more or less likely to be lost from the population than non-beneficial ones due to bottlenecks, and it is thus the selection pressure which will add this advantage.

There are already examples of adaptive evolution strategies in mammalian cell cultures employed to generate cell lines of increased industrial interest or in order to better understand a particular metabolic question. For example, researchers in the late nineties used successive rounds of protein deprivation in culture media combined with over-expression of cyclin E to develop Chinese Hamster Ovary (CHO) cells that could be grown in suspension culture, a development that has had major implications in industrial MAb production (Bailey *et al*, 1996). Furthermore, Spitz *et al* developed a Chinese hamster fibroblast cell line with increased catalase activity and thus greater productivity through over-exposure to Hydrogen Peroxide (Spitz *et al,* 1988), as demonstrated by

analytical work by Keightley's group (Keightley *et al,* 2003), and work by K. Syddall in the David James' group growing successive CHO generations in reduced temperatures produced cells that were far larger, and with greater production characteristics than the original industrial cell line (James, 2016).

Chapter Summary

Production of biopharmaceuticals using CHO cells continues to be a lucrative and expanding industry, with global profits in the multi-million dollar scale. However, with many proprietary products now coming off patent, and with novel drug targets increasingly moving away from the easily expressed Monoclonal Antibodies and towards more difficult to express proteins, production companies continue to invest extensively in cell line, media and process development. Whilst commercially available Chemically Defined media served a vital purpose in ensuring that biologics could be produced quickly and with regulatory approval, there has been a shift towards developing novel media to meet the differing demands of each new biologic.

There has been a growing body of work indicating the oxidative stress and the accumulation of Reactive Oxygen Species has a direct and detrimental impact on producer cell productivity. Despite mixed results in *in vivo* testing and clinical trials, there are a range of anti-oxidant chemicals that can be used successfully *in vitro* to combat ROS directly or to support and enhance the anti-oxidant defences already present within the cell. It is known that there are already anti-oxidant chemicals within chemically defined media, but the types and concentrations used remain closely guarded secrets. It is also clear from the body of work surrounding anti-oxidants in medical research that many are tissue specific, and all are concentration sensitive, and so the anti-oxidants used in CD media may have little to no effect on the oxidative capacity of cultured cells.

Of the various engineering strategies available to cell line development, directed evolution is one of the oldest and in many ways perhaps one of the simplest to implement. Experimental Microbial Evolution (EME) has a long history in microbiological research investigating population dynamics, but was not recognised by wider parts of the life science field as the powerful tool it was until much later. Since then, however, it has become one of the two predominant methods for cell line development, along with the more knowledge intensive rational design.

Chapter 3:
Materials and Methods

Contents

List of Abbreviations

CHO	Chinese Hamster Ovary
rMAb	Recombinant Monoclonal Antibody
AHC	Apollo Host Cell Line
APC	Apollo Producer Cell Line
VCD	Viable Cell Density
IVCD	Integral Viable Cell Density
μ	Cell Growth
PBS	Phosphate Buffered Saline
DMSO	Dimethyl Sulfoxide
q_P	Cell Specific Productivity
PFA	Para-formaldehyde
q_G	Cell Specific Glucose Consumption
q_L	Cell Specific Lactate Production
ROS	Reactive Oxygen Species
H_2O_2	Hydrogen Peroxide
IC_{50}	50% Inhibitory Concentration
IC_{75}	75% Inhibitory Concentration
GN	Generation Number
DE	Directly Evolved Cell Line
CTL	Co-Cultured Control Cell Line

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3.1 Introduction

As with any scientific endeavour, it is important to accurately detail the methods employed for the duration of this work in order to enable reproducibility should any other investigator wish to review any part of it. The following chapter details all aspects of the study, from basic cell culture techniques employed, to use of specific assays or equipment, to the lineage of the cell lines utilised, with reference to any appendices as appropriate.

3.2 Cell Line Characterisation

3.2.1 Cell lines

Suspension adapted Chinese Hamster Ovary (CHO) DG44 derived host cell line (C2A), and two producer cell lines (7C3, 6A8; established from C2A stable pools) producing a recombinant Monoclonal Antibody (rMAb) were provided by FUJIFILM Diosynth Biotechnologies (FUJIFILM, Billingham, UK), and were used for all experimentation conducted on site at the University of Sheffield, UK. Apollo™ host (AHC) and producer (APC) cell lines were used in addition to the other three cell lines for experimentation conducted on site at FUJIFILM.

3.2.2 Maintenance and routine sub-culture

Where cell samples needed to be taken, or sterility was required for experimental work, this was conducted in a class II Type B1 Biological Safety Cabinet using standard sterile techniques. Sterile sub-culture media and supplements were obtained from ThermoFisher Scientific, Paisley, UK. Host cell lines C2A and AHC were cultured in Gibco® CD DG44 medium supplemented with 8mM Gibco® L-Glutamine and 0.18 % Gibco® Pluronic F68. Producer cell lines 7C3 and 6A8 were cultured in GIbco® CD Opti-CHO medium supplemented with 8mM Gibco® L-Glutamine and 0.1mg/mL Hygromycin B. Producer cell line APC was cultured in Gibco® CD Opti-CHO medium supplemented with 8mM Gibco® L-Glutamine and 75nM Methotrexate (Sigma-Aldrich Ltd, Dorset, UK).

Viable Cell Density (VCD) and percentage viability in all cases were determined through Trypan Blue exclusion using Vi-CELL® Cell Viability Analyser (Beckman Coulter, High Wycombe, UK). Cell samples at a volume of 600 µL were analysed. Gibco® Dulbecco's Phosphate Buffered Saline (PBS) was used where necessary for sample dilution immediately prior to analysis, but samples were never diluted by more than a factor of 3. Integral Viable Cell Density (IVCD, cell h mL^{-1}) was calculated according to equation 3.1, and specific growth rate (µ, h^{-1}) was calculated according to equation 3.2.

$$IVCD_n = \left(\frac{(VCD_{Tn}+VCD_{Tn-1})\times(\Delta T)}{2}\right) + IVCD_{n-1} \qquad \textbf{Equation 3.1}$$

$$\mu = ln\left(\frac{(VCD_{Tn}/VCD_{Tn-1})}{(\Delta T)}\right)$$ **Equation 3.2**

Where VCD_{tn} is the Viable Cell Density ($x10^6$ cells mL^{-1}) at a given timepoint and T_n is the timepoint (h) and ΔT is the change in time since the previous timepoint.

Upon revival from cryopreservation, VCD was determined and culture volume adjusted to achieve a VCD of 0.3 x 10^6 cells mL^{-1}. Cells were then sub-cultured every 3 days (C2A, AHC) or every 3-4 days (7C3, 6A8, APC), seeded at a density of 0.2 x 10^6 cells mL^{-1}. Cells grown at the University of Sheffield were incubated in an orbital throw incubator (INFORS-HT, Switzerland) in either Erlenmeyer Flasks with Vent Cap (Corning®, Tewksbury, US) at 140 rpm at a volume not exceeding 1/5 total flask volume, or 50 mL vented Bioreactor Cultiflasks (Sartorius AG, Göttingen, Germany) at 170 rpm at 10-15 mL culture volume, both at 37 °C and with 5 % (v/v) CO_2. Cells grown at Fujifilm Diosynth Biotechnologies were incubated in a Certomat CT incubator (Sartorius AG, Göttingen, Germany) in either Erlenmeyer Flasks with Vent Cap (Corning®, Tewksbury, US) at 140 rpm at a volume not exceeding 1/5 total flask volume, or 50 mL vented Bioreactor Cultiflasks (Sartorius AG, Göttingen, Germany) at 200 rpm at 15 mL culture volume, both at 36.5 °C, 5 % (v/v) CO_2 and 80 % humidity. Batch cultures of C2A ran for no more than 6 days, and of 7C3 and 6A8 for no more than 8 days, without feeding.

3.2.2.1 Fed-batch Feeding Strategy: Sheffield

Fed-batch cultures of 7C3 and 6A8 ran for 11 days or until Viability was less than 70 % and were fed on days 3, 6 and 9 with a 1:1 mix of CHO CD Efficient Feed™ A and B (Invitrogen™, ThermoFisher, Paisley, UK) at a volume of 10 % of the cell culture volume (eg. For a 30 mL culture volume, 3 mL of 1:1 Feed A: Feed B would be added on each feed day).

3.2.2.2 Fed-batch Feeding Strategy: FUJIFILM Diosynth Biotechnologies

Fed-batch cultures of APC, 7C3 and 6A8 ran for 12 days or until Viability was less than 70 % and were fed on days 3, 5, 7, 10 and 12 with Acti-CHO Feed A at 4 % cell culture volume and Acti-CHO Feed B at 0.4 % cell culture volume (both GE Healthcare Life Sciences, Buckinghamshire, UK) (eg. For a 30 mL culture volume, 1.2 mL Feed A and 0.12 mL Feed B would be added on each feed day). Cultures were also supplemented with 6 mM Gibco® L-Glutamine (ThermoFisher Scientific, Paisley, UK) on days 3, 5 and 7.

3.2.3 Cryopreservation

Cells were harvested during mid-exponential growth (usually day 3) when culture viability was >90 %. Viability and VCD was determined and the volume of freezing medium needed to reach a final cell density of 1 x 10^7 viable cells mL^{-1} was then calculated using equation 3.3.

$$Volume\ culture\ required = \frac{Number\ of\ cells\ per\ cryovial \times Number\ of\ cryovials}{Viable\ Cell\ Density\ from\ shake\ flask}$$ **Equation 3.3**

Freezing medium was prepared by adding 10 % (v/v) Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich Ltd, Dorset, UK) to fresh 4 °C culture medium. Cells were pelleted by centrifugation at 1800 x *g* for 5 minutes and the supernatant aspirated. Cell pellets were then re-suspended in the predetermined volume of freezing medium, and 1.5 mL aliquots dispensed into 2 mL round bottomed cryovials (Fisher Scientific Ltd, Loughborough, UK). Cryovials were placed in a Nalgene® Mr. Frosty and placed in a -80 °C freezer overnight, before being transferred to liquid nitrogen for storage.

3.2.4 Cell Revival

Cryovials were removed from liquid nitrogen and warmed in a sterile water bath at 37 °C until only a small volume remained frozen, and then removed from the water bath and sprayed down with 70 % (v/v) ethanol (Crystel, Snailwell, UK), placed in the Class II B1 Biological Safety cabinet and allowed to air dry. Thawed cell suspension was then transferred to a 125 mL vent capped Erlenmeyer Flask (Corning®, Tewksbury, US) and 20mL fresh culture medium was added drop-wise over 1 minute. Cell density was assessed using a Vi-CELL® Cell Viability Analyser (Beckman Coulter, High Wycombe, UK) according to manufacturer's instructions and an appropriate volume of culture medium was added to reach a seeding density of 0.3 x 10^6 cells mL^{-1}, according to Equation 3.4. Cells were then incubated as described previously.

$$Volume = \left(\frac{VCD_A \times Seeding\ volume}{VCD_D}\right) - Seeding\ Volume$$ **Equation 3.4**

Where Seeding Volume is the initial 20 mL media added, VCD_A is the measured Viable Cell Density post-thaw, and VCD_D is the desired final Viable Cell Density.

3.2.5 Productivity

3.2.5.1 IgG Sample Acquisition

Aliquots of 200 µL were taken from 7C3 and 6A8 cultures daily and cells pelleted by centrifugation at 180 x g for 5 minutes. Supernatants were aspirated and retained in 0.5 mL Eppendorf tubes (Eppendorf UK Ltd, Stevenage, UK), and placed in storage at -20 °C. Cell pellets were discarded.

3.2.5.2 IgG Quantification: Octet

Supernatant samples were thawed at room temperature (RT) ready for analysis. Samples were diluted by a factor of 20 in cell culture medium and then dispensed into black, flat bottomed 96-well

plates at a volume of 200 µL. Rows 11 and 12 of the plates were reserved for 200 µL per well of regeneration buffer (0.375 g Glycine in 500 mL deionized water, pH 1.0) and cell culture medium respectively. One row of Octet® biosensor tips (PALL FortéBio, Southampton, UK) per plate were incubated in cell culture medium for ten minutes prior to analysis. Plates were then analysed on the Octet® HTX System (PALL FortéBio, Southampton, UK) according to the manufacturers' instructions. Data was exported into Microsoft Excel and analysed using GraphPad Prism.

3.2.5.3 IgG Quantification: Fluorescence Polarisation

Supernatant samples were thawed at RT ready for analysis. Samples were diluted by a factor of 16 in cell culture medium and dispensed into Valitacell 96 well plates, followed by Pierce™ Protein G IgG Binding Buffer (ThermoFisher Scientific, Paisley, UK) with 1.28 mg/mL Bovine Serum Albumin (BSA) at a 1:1 ratio. Plates were incubated for 30 mins at room temperature protected from light, then analysed for Fluorescence Polarisation using a PheraSTAR Plus plate reader (BMG Labtech, Aylesbury, UK). Data was exported into Microsoft Excel and analysed using GraphPad Prism.

3.2.5.4 Cell Specific Productivity

Cell Specific Productivity (q_P) was calculated according to Equation 3.5.

$$q_P = \frac{Product\ Titre}{IVCD} \qquad \textbf{Equation 3.5}$$

Where IVCD is the Integral Viable Cell Density at a given time-point, as calculated by Equation 3.1.

3.2.6 Reactive Oxygen Species quantification

3.2.6.1 Cell Staining and Fixing

Viable Cell Density (VCD) of C2A, 6A8 or 7C3 cell cultures was determined using a ViCell® Cell Viability Analyser (Beckman Coulter, High Wycombe, UK) and aliquots were taken, and adjusted with culture medium to generate 1 mL aliquots of cells at a density of 1×10^6 cells mL^{-1}. 450 µL Dimethyl Sulfoxide (Sigma-Aldrich, Dorset, UK) was added to one vial of CellROX® Deep Red Reagent (Life Technologies™, Paisley, UK) to yield a concentration of 250 µM dye. 2 µL of this stock solution was added to the 1 mL cell aliquots to yield a final concentration of 0.5 µM dye per sample. Two control samples were taken; the first control sample was treated in the same way as all other samples but without addition of any dyes as a negative control to establish any auto-fluorescence; the second sample was taken from late stage culture and stained only with CellROX® Deep Red as a positive stain for Reactive Oxygen Species.

Cell samples were incubated for 30 minutes at 37 °C, protected from light, before being washed once with Phosphate Buffered Saline (PBS; Sigma-Aldrich, Dorset, UK). Cells were resuspended in fridge cold 4 % paraformaldehyde (PFA; Sigma-Aldrich, Dorset, UK) to a

concentration of 1 x10^7 cells mL^{-1} and incubated at 4 °C for 15 minutes. After incubation in PFA, cells were washed once with PBS and then resuspended in PBS to a concentration of 1 x10^6 cells mL^{-1}. Fixed cell samples were stored at 4 °C, protected from light, until analysis on the flow cytometer (3.1.6.2 "Imaging using Flow Cytometry").

3.2.6.2 Imaging using Flow Cytometry

Fixed cell samples were centrifuged at 1800 x g for 5 minutes, and then re suspended in 200 µL of PBS before being transferred to a clear, flat bottomed 96 well plate (STARLAB (UK) Ltd, Milton Keynes, UK). Cell samples were analysed using an Attune® Autosampler flow cytometer (ThermoFisher Scientific, Paisley, UK) using the RL1-A filter. Instrument settings and gating strategy were employed as per Appendix A. Flow Cytometry Standard (FCS) files were analysed using the Attune® Software and statistics generated were analysed using Microsoft Excel and GraphPad Prism.

3.2.7 Glucose and Lactate Determination:

Aliquots of 500 µL were taken daily (every 24 h) from C2A batch, and 7C3 and 6A8 fed-batch cell cultures and centrifuged at 180 x g for 5 minutes. Supernatant samples were stored at -20 °C until analysis, cell pellets were discarded. After thawing to room temperature, supernatant samples were analysed using the Cedex Bioanalyser (Roche, Burgess Hill, UK) for glucose and lactate concentration, according to the manufacturer's instructions. The rates of change in supernatant glucose concentration were calculated according to Equations 3.6 and 3.7.

$$Glucose\ Consumption\ Rate = -\left(ln\left(\frac{([G]_{Tn}/[G]_{Tn-1})}{(\Delta T)}\right)\right) \qquad \textbf{Equation 3.6}$$

Where $[G]_{Tn}$ is the glucose concentration (mg/L) at a given timepoint, $[G]_{Tn-1}$ is the glucose concentration (mg/L) at the previous timepoint and ΔT is the change in time (h) between the two.

$$Lactate\ Production\ Rate = ln\left(\frac{([L]_{Tn}/[L]_{Tn-1})}{(\Delta T)}\right) \qquad \textbf{Equation 3.7}$$

Where $[L]_{Tn}$ is the lactate concentration (mg/L) at a given timepoint, $[L]_{Tn-1}$ is the lactate concentration (mg/L) at the previous timepoint and ΔT is the change in time (h) between the two. Rate of concentration change was then correlated with cell growth as determined by the IVCD, to reveal the cell specific consumption of glucose (q_G) and the cell specific production of lactate (q_L). Equations 3.8 and 3.9 indicate how q_G and q_L were calculated.

$$q_G = -\left(\frac{\left(\frac{[G]_{Tn}-[G]_{Tn-1}}{IVCD_{Tn-1}}\right)}{\Delta T}\right)$$ **Equation 3.8**

$$q_L = \frac{\left(\frac{[L]_{Tn}-[L]_{Tn-1}}{IVCD_{Tn-1}}\right)}{\Delta T}$$ **Equation 3.9**

Data was analysed using Microsoft Excel and statistical comparison and figures created using GraphPad Prism.

3.3 High Throughput Chemical Screening

3.3.1 Chemicals used for screening

Anti- and Pro-oxidants used and the concentration range used in screenings are detailed in Table 3.1.

Table 3.1. Anti-Oxidant and Pro-Oxidant chemicals screened using high-throughput multi-well methods.

Chemical	Abbreviation	Concentration Range	Solvent	Supplier
Ascorbic Acid	AA	0.01 - 5 mM	Culture Medium	Acros Organics, Cat#:105021000
α-lipoic Acid	AL	0.1 - 50 mM	Ethanol to 1 M, serial dilutions in Culture Medium	Sigma, Cat#:T5625
Butylated Hydroxyanisole	BHA	0.1 - 50 µM	Ethanol to 0.5 M, serial dilutions in Culture Medium	Acros Organics, Cat#:235231000
Glutathione	GSH	1 - 500 µM	Culture Medium	Sigma, Cat#:G4251
L-Carnosine	LCA	0.1 - 100 µM	Culture Medium	Sigma, Cat#: C9625
MitoQ	MQ	0.5 - 100 nM	Culture Medium	Prof. M. Murphy, University of Cambridge, UK
MitoTEMPO	MT	0.5 - 50 nM	Culture Medium	Sigma, Cat#:SML0737
N-Acetylcysteine	NAC	1 - 10 mM	Culture Medium	Sigma, Cat#: A9165
L-2-Oxothiazolidine-4-carboxylic acid	OTZ	1 - 10 mM	Culture Medium	Sigma, Cat#:O6254
Sodium Selenite	SS	5 - 75 nM	Culture Medium	Sigma, Cat#:S5261
Buthionine Sulfoximine	BSO	10 - 1000 µM	Culture Medium stock at 100 mM	Sigma, Cat#:B2515
2-Deoxy-d-Glucose	2DDG	0.1 - 2.5 mM	Culture Medium stock at 100 mM	Alfa Aesar, Cat#:L07738
Hydrogen Peroxide	H_2O_2	0.01 - 2.5 mM	Water to 0.5 M, serial dilutions in Culture Medium	Sigma, Cat#:H1009

Menadione	ME	1 - 500 nM	DMSO to 50 mM, serial dilutions in Culture Medium	Sigma, Cat#:M5750

3.3.2 Presto-Blue® Analysis

Chemicals to be screened were added to the wells of a clear, flat bottomed CELLSTAR® 96 well plate (Greiner Bio One, Stonehouse, UK) in appropriate volumes, not exceeding 10 µL. Cells from C2A, 6A8 or 7C3 cultures were seeded in culture medium at a volume of 90 µL per well and density of 0.2 x10^6 cells mL^{-1}, shaken at 700 rpm for 20 secs and incubated for 3 days at 37 °C, 5% v/v CO_2, and 70 % humidity.

A 1:1 solution of PrestoBlue® Cell Viability Reagent (ThermoFisher Scientific, Paisley, UK) and culture medium was prepared and allowed to equilibrate to room temperature. On day 3 of incubation, 20 µL of this solution was added to each well. Plates were shaken at 700 rpm for 20 secs, incubated at 37 °C for 30 mins, shaken again and fluorescence intensity at 585 nm measured using an Omega fluorimeter (BMG Labtech, Aylesbury, UK). Where indicated, plates were occasionally imaged using a CloneSelect™ Imager (CSI; Molecular Devices, Wokingham, UK) prior to PrestoBlue addition.

3.3.3 CellTOX™ Green Cytotoxicity Analysis

Chemicals to be screened were added to the wells of a clear, flat bottomed CELLSTAR® 96 well plate (Greiner Bio One, Stonehouse, UK) in appropriate volumes, not exceeding 10 µL. Cells were seeded in culture medium at a volume of 100 µL per well and density of 0.2 x10^6 cells mL^{-1} and shaken at 700 rpm for 20 secs. After 20 minutes incubation at 37 °C to allow cells to settle, plates were imaged using the Clone Select Imager (CSI; Molecular Devices, Wokingham, UK). Plates were incubated for 3 days at 37 °C, 5 % v/v CO_2 and 70 % humidity, being shaken and imaged once daily during this period.

CellTOX™ Green Cytotoxicity (Promega, Southampton, UK) 2X Reagent and Dye were prepared according to the manufacturer's instructions. After final round of shaking and imaging on day 3, 100 µL of the CellTOX™ Green Reagent and Dye mix were added per well of the plate. Plates were shaken at 700 rpm for 20 seconds and incubated at room temperature for 15 mins, protected from light. Fluorescence intensity at 520 nm was measured using a PheraSTAR Plus plate reader (BMG Labtech, Aylesbury, UK).

3.4 Batch and Fed-Batch screening of Anti- and Pro- Oxidants

3.4.1 Culture Conditions

Batch culture experiments were conducted in Sheffield. Producer cell lines 7C3 and 6A8 were cultured in medium as previously described (3.2.2 "Routine Sub-Culture and Maintenance"). For growth and productivity analysis, cells were cultured at 15 mL volumes in 50 mL vented Bioreactor Cultiflasks (Sartorius AG, Göttingen, Germany) at 170 rpm, 37 °C and 5 % v/v CO_2. For Reactive Oxygen Species (ROS) level analysis with flow cytometry, cells were cultured at 30 mL volume in E125 Erlenmeyer Flasks with Vent Cap (Corning®, Tewksbury, US) at 140 rpm, 37 °C, 5 % v/v CO2 and 70 % humidity. Anti-oxidant chemicals were included in the basal medium according to Table 3.2.

Fed-batch shaking flask cultures were conducted in Billingham. Producer cell lines APC, 7C3 and 6A8 were cultured and fed according to the regimes previously described (3.2.2 "Routine Sub-Culture and Maintenance"), at 30 mL culture volume in E125 Erlenmyer Flasks with Vent Cap (Corning®, Tewksbury, US). Anti-oxidant chemicals were either included in the basal medium or in the feed as dictated in Tables 3.3 and 3.4.

Table 3.2. Concentrations of Anti-Oxidant chemicals introduced to cell culture based on data from combined CellTOX and CSI assays.

Anti-Oxidant Compound	Abbreviation	Concentration Used
Ascorbic Acid	AA	100 µM
α-Lipoic Acid	AL	25 µM
Butylated Hydroxyanisole	BHA	1 µM
Glutathione	GSH	50 µM
L-Carnosine	LCA	10 mM
MitoQ	MQ	5 nM
MitoTEMPOL	MT	10 nM
N-Acetylcysteine	NAC	1 mM
Procysteine	OTZ	1 mM
Sodium Selenite	SS	100 nM

Table 3.3. Varying combinations of Anti-Oxidant chemicals were introduced to cell culture. Those included in the Basal Supplement were added to culture on Day1. Those included in the Feed Supplement were added to culture at the same time as Acti-CHO Feeds A and B on Days 3, 5, 7, 10 and 12.

CODE	CELL LINE	BASAL SUPPLEMENT	CONCENTRATION	FEED SUPPLEMENT	CONCENTRATION
A	7C3	Glutathione	50 µM	MitoTEMPO	5 nM
B	7C3	L-Carnosine	10 mM	MitoTEMPO	5 nM
C	7C3	L-Carnosine	10 mM	α-Lipoic Acid	25 µM
D	7C3	L-Carnosine + Glutathione	10 mM/50 µM	MitoTEMPO	5 nM
E	7C3	L-Carnosine + Glutathione	10 mM/50 µM	--	N/A
F	7C3	Ascorbic Acid	100 µM	N-Acetylcysteine	1 mM
G	6A8	Glutathione	50 µM	Butylated Hydroxyanisole	1 µM
H	6A8	L-Carnosine + Glutathione	10 mM/50 µM	--	N/A
I	6A8	α-Lipoic Acid	25 µM	Sodium Selenite	100 nM
J	6A8	L-Carnosine	10 mM	MitoQ	10 nM
K	6A8	L-Carnosine	10 mM	α-Lipoic Acid	25 µM
L	6A8	Glutathione + MitoTEMPO	50 µM/5 nM	--	N/A
M	APC	Glutathione + MitoTEMPO	50 µM/5 nM	--	N/A
N	APC	L-Carnosine + Glutathione	10 mM/50 µM	--	N/A
O	APC	α-Lipoic Acid	25 µM	Sodium Selenite	100 nM
P	APC	Ascorbic Acid	100 µM	N-Acetylcysteine	1 mM
Q	APC	L-Carnosine	10 mM	α-Lipoic Acid	25 µM
R	APC	L-Carnosine	10 mM	MitoTEMPO	5 nM
S	APC	L-Carnosine	10 mM	MitoQ	10 nM
T	APC	Glutathione	50 µM	MitoQ	10 nM
U	APC	Glutathione	50 µM	MitoTEMPO	5 nM
CTL	ALL	--	N/A	--	N/A

Table 3.4. Combinations of Anti-Oxidant chemicals were introduced to cell culture. The same combinations were used for all three cell lines. Chemicals in "Total Feed Supplement" were added at the same time as every feed with Acti-CHO Feeds A and B on Days 3, 5, 7, 10 of culture. Chemicals in "Late Stage Feed Supplement" were added with Acti-CHO Feeds A and B on Days 7 and 10 of culture.

CODE	TOTAL FEED SUPPLEMENT	CONCENTRATION	LATE STAGE FEED SUPPLEMENT	CONCENTRATION
α	L-Carnosine	10 mM	MitoTEMPO	5 nM
β	L-Carnosine	10 mM	α-Lipoic Acid	25 µM
γ	Glutathione	50 µM	MitoTEMPO	5 nM
δ	Glutathione	50 µM	--	N/A
ε	α-Lipoic Acid	25 µM	Sodium Selenite	100 nM
ζ	MitoTEMPO	5 nM	--	N/A
η	Ascorbic Acid	100 µM	N-Acetylcysteine	1 mM
CTL	--	N/A	--	N/A

3.4.2 Productivity

Product samples were acquired and analysed as described previously (3.2.5 "Productivity"). For Batch experiments, supernatant samples were acquired on Day 4, 5, 6, 7, 8. For Fed-Batch experiments, supernatant samples were acquired on Day 5, 7, 10 and 12.

3.4.3 Reactive Oxygen Species quantification

3.4.3.1 Cell Staining and Fixing

Staining and fixing were performed as described previously (3.2.6.1 "Cell Staining and Fixing"), with one additional staining step. After staining with CellROX Deep Red and washing, cells were resuspended in 1 mL PBS and 1 µL LIVE/DEAD® Fixable Green Dead Cell Stain (Life Technologies™, Paisley, UK) added. Samples were incubated at room temperature for 30 mins, protected from light. Cells were then pelleted and fixed as described previously (3.2.6.1 "Cell Staining and Fixing"). The two control samples described previously were taken, with an additional third sample which was heat treated at 60°C for 20 mins before staining and then only stained with LIVE/DEAD® Fixable Green Dead Cell Stain (Life Technologies™, Paisley, UK), as a positive control for dead cells. For batch experiments, cell samples were taken on Day 5 and 7. For Fed-Batch experiments, cell samples were taken on Day 6 and 11.

3.4.3.2 Imaging using Flow Cytometry.

Fixed cell samples acquired at the University of Sheffield were analysed as described previously (3.2.6.2 "Imaging Using Flow Cytometry"), but both the RL1-A and BL1-A filters were used to take into account the addition of the LIVE/DEAD® stain. Instrument settings and gating strategy were employed as per Appendix A. FCS files were analysed using the Attune® Software and statistics generated were analysed using GraphPad Prism.

Fixed cell samples acquired at Fujifilm Diosynth Biotechnologies were transferred to 5mL BD Falcon™ Round Bottom Tubes and analysed using a FACSAria II Flow Cytometer (BD Biosciences, Plymouth, UK) using the FITC-A and APC-A filters to detect the LIVE/DEAD Green Fixable Stain (Life Technologies, Paisley, UK) and CellROX Deep Red stain (Life Technologies, Paisley, UK), respectively. Instrument settings and gating strategy are detailed in Appendix A. FCS files were analysed using the Attune® Cytometric Software (Applied Biosystems®, California, US) and statistics generated were analysed using GraphPad Prism.

3.4.5. Design of Experiments

$IVCD_{MAX}$, final day product titre, final day cell specific productivity, and ROS content in late-stage culture for all anti-oxidant batch cultures were fed into a Design of Experiments (DoE) model in order to predict the outcomes of feeding strategies. Data taken from cultures treated on Day0 and Day4 and from both 7C3 and 6A8 cell lines were included. The DoE was run using all possible combinations of these factors resulting a general factorial DoE. The main effects of the factors and their interactions could be estimated independently due to the design being complete. Data was analysed and models were created using JMP 12 software. All DoE work was conducted by the in-house bioinformatician Gwen Ninon at FUJIFILM's Billingham site.

3.5 Directed Evolution of Host Cell Lines

3.5.1 Evolution strategy.

Host cells (C2A) were maintained at 30 mL volumes in cell culture medium and conditions as previously described (3.2.2 "Routine Sub-culture and maintenance"). Triplicate flasks were maintained under evolution pressure conditions, in addition to triplicate flasks that were maintained to the same generation number but without evolution pressure. Evolution pressure used was Hydrogen Peroxide (H_2O_2).

The half maximal inhibitory concentration (IC_{50}) of H_2O_2 was determined through high-throughput screening with Presto Blue viability reagent as described previously (3.3.2 "Presto Blue Analysis"). H_2O_2 was included in the culture medium at 250 µM (IC_{50}) to C2A Host Cell cultures. With

each subculture, fresh H_2O_2 was added at the same concentration until cell viability recovered to ≥80 %, as determined by analysis with a ViCell® Cell Viability Analyser (Beckman Coulter, High Wycombe, UK).

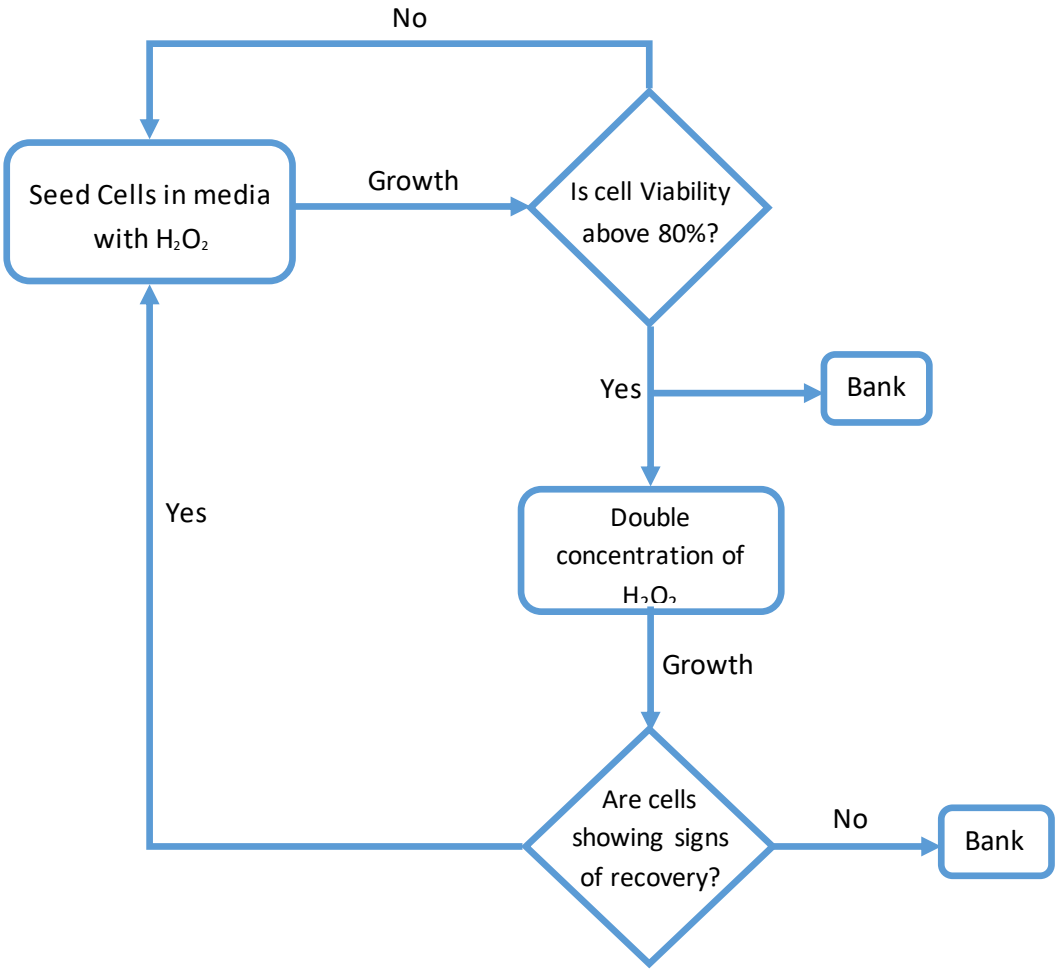

Fig. 3.1. Decision Diagram for evolution process with H_2O_2

This process was repeated until concentrations were reached at which point the cells were no longer able to recover (1.6 mM H_2O_2). Cells were cryopreserved before each concentration increase, and cell banks were created at the end of the evolution period (8 vials cryopreserved for all 3 new cell lines, as described previously (3.2.3 "Cryopreservation")). Post revival of banked cell lines, H_2O_2 was not included in cell culture medium. In order to comply with industry standards, revived cells were not sub-cultured more than 20 times during experimentation.

3.5.2 Calculation of Generation Number

Generation Number (GN) was calculated according to Equation 3.10.

$$GN = \log 2\left(\frac{VCD_{Day0}}{VCD_{Day3}}\right) + GN_{-1} \qquad \textbf{Equation 3.10}$$

Where VCD is the Viable Cell Density on the day of seeding (Day0) and on the day of sub-culture (Day3) and GN_{-1} is the generation number at the previous sub-culture.

3.5.3 Transient Transfection Optimisation

C2A cells were used for optimisation of transfection protocol using the 4D-Nucleofector™ System (Lonza, Cambridge, UK). Cells were cultured as previously described, with cell culture volume being expanded to 100 mL (in an E500 Erlenmyer Flask with Vent Cap (Corning®, Tewksbury, US)). Product vector was prepared by Fujifilm Diosynth Biotechnologies R&D department. Prior to transfection, 4D-Nucleofector™ SF Solution and Supplement were allowed to equilibrate to room temperature before being mixed together at a ratio of 4.5:1, respectively.

On day 2 of culture, VCD was determined using a Vi-CELL® Cell Viability Analyser (Beckman Coulter, High Wycombe, UK) and "master mixes" of cells, product vector and 4D-Nucleofector™ SF solution were prepared according to Table 3.4.

Table 3.4. Master Mix compositions for 4D-Nucleofector™ transfection optimisation.

Master Mix	Cell Density (x10^6 cells mL^{-1})	DNA Quantity (µg)	Volume Nucleofector™ SF Solution (µL)
A	16	22	320
B	16	15	320
C	9.6	22	320
D	9.6	15	320
E	3.2	22	320
F	3.2	15	320

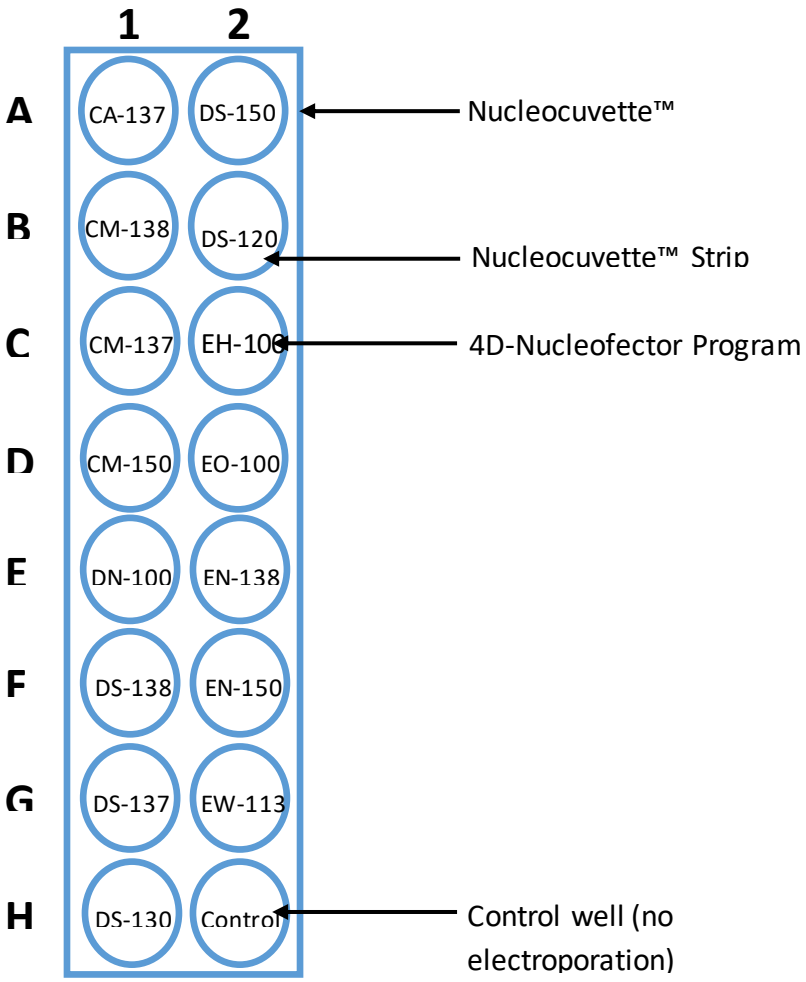

Figure 3.2. 4D-Nucleofector Program Optimisation Protocol

20 µL of master mix was added to each well of a Nucleocuvette™ Strip, with one strip used per master mix. Inoculated Nucleocuvette™ Strip was then inserted to the 4D-Nucleofector and transfection was conducted according to manufacturer's instructions, using the protocol detailed in Figure 3.2 ("Amaxa™ 4D-Nucleofector™ Optimization Protocol for Cell Lines", Lonza Cologne GmbH, 2010).

After transfection, strips were incubated at room temperature for ten minutes, and then 80 µL recovery medium (Gibco® CD Opti-CHO with 8mM Gibco® L-Glutamine and 1X HT Supplement (all ThermoFisher Scientific, Paisley, UK)) was added per well. Medium and cells were mixed gently by pipetting, and then 50 µL were transferred to a 24-well plate prepared as per Figure 3.3. Supernatant samples at 50 µL were taken on Day 6 and analysed for productivity using the Octet® HTX System as described previously (3.2.5.2 "IgG Quantification: Octet").

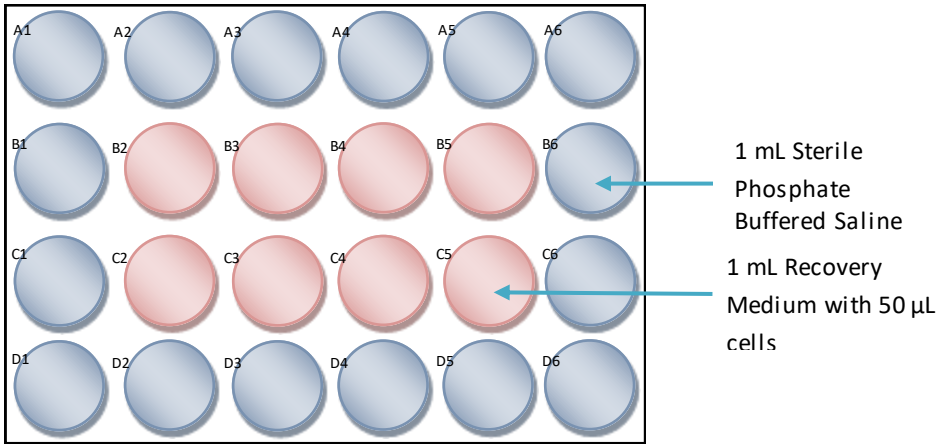

Figure 3.3. Plate layout for post-transfection culture during transient transfection optimisation.

3.5.4. Transient Transfection

3.5.4.1. Transfection Procedure

Prior to transfection, 4D-Nucleofector™ SF Solution and Supplement were allowed to equilibrate to room temperature before being mixed together at a ratio of 4.5:1, respectively.

On Day2 of culture, C2A host cells, evolved cell lines (DE), co-cultured cell lines (CTL) and Apollo Host cells (AHC) were analysed for Viability and VCD as described previously ("3.2.2 Maintenance and routine sub-culture"). Appropriate aliquots were taken to achieve a cell quantity of 3 x 10^6 cells per transfection and centrifuged at 180 *x g* for 5 minutes. Supernatent was discarded, and cells were resuspended in 4D-Nucleofector™ SF Solution : Supplement mix, at a volume of 20 µL per transfection. Circular plasmid DNA containing a recombinant MAb was then added to the mix at a volume of 7 µL per transfection. This master mix was mixed gently by pipetting.

100 µL master mix was placed into a Nucleocuvette™ Vessel and the cell suspension was electroporated using a 4D-Nucleofector™ set to program EH-100. Immediately post electroporation, 500 µL recovery medium (Gibco® CD Opti-CHO with 8 mM Gibco® L-Glutamine and 1X HT Supplement (all ThermoFisher Scientific, Paisley, UK)) was added to the Nucleocuvette™ Vessel and incubated at RT for 10 minutes. Subsequent to incubation, cell suspension was divided between 2 wells (300 µL per well) of a 6-well CELLSTAR Cell Culture Multiwell Plate (Greiner Bio-One) containing 2 mL pre-warmed recovery medium per well. Plates were incubated in an MCO-20AIC static CO_2 incubator (SANYO Electric Biomedical Co., Ltd., Loughborough, UK) at 36.5 °C, 5 % CO_2 and 80 % humidity.

3.5.4.2. Reactive Oxygen Species and IgG Determination

On Day 3, 200 µL supernatant was taken from half the wells and stored at -20 °C until IgG quantification analysis. The wells were then mixed gently by pipetting to dislodge cells from the well bottom, and a 200 µL aliquot was taken to be analysed for Viability and VCD (sample diluted with 400 µL PBS) using a Vi-CELL® Cell Viability Analyser (Beckman Coulter, High Wycombe, UK) . An aliquot was also taken and stained and fixed for ROS analysis as described previously ("3.3.3.1 Cell Staining and Fixing"). Plates were returned to the incubator, and this sample acquisition process was repeated with the other half of the wells on Day 6 of culture.

IgG quantification was conducted with thawed samples as described previously ("3.2.5.3 IgG Quantification: Fluorescence Polarisation"). Stained and fixed cell samples were analysed using the FACSAria II (BD Biosciences, Plymouth, UK) as described previously ("3.3.3.2 Imaging using Flow Cytometry.").

3.5.5. Stable Transfection

3.5.5.1 Transfection Procedure

Evolved C2A and original C2A cells were transfected as described previously (3.4.4.1 "Transfection Procedure") but instead of cells being transferred post-transfection to 6 well plates, they were transferred to 75 cm^2 Nunc™ Cell Culture Treated Flasks with Filter Caps ("T75 Flasks", ThermoFisher Scientific, Paisley, UK) (four cuvettes per cell line, one flask per cuvette) containing 20 mL pre-warmed recovery medium (Gibco® CD Opti-CHO with 8 mM Gibco® L-Glutamine and 1X HT Supplement (all ThermoFisher Scientific, Paisley, UK)).

Transfected cells were incubated for 24 h in an MCO-20AIC static CO_2 incubator (SANYO Electric Biomedical Co., Ltd., Loughborough, UK) at 36.5 °C, 7.5 % CO_2 and 80 % humidity. Culture volume was then transferred to a 50 mL Falcon™ Centrifuge Tube (Corning®, Tewksbury, US) and centrifuged at 180 x *g* for 5 minutes. Supernatent was discarded, cells re-suspended in 20 mL selection medium (Gibco® CD Opti-CHO with 8 mM Gibco® L-Glutamine (both ThermoFisher Scientific, Paisley, UK) and 75 nM Methotrexate (Sigma-Aldrich Ltd, Dorset, UK)) and transferred back to the T75 Flasks. Cells were incubated in a static incubator in the same conditions as before for 18 days.

On Day18, 200 µL aliquots were taken from each flask, diluted with 400 µL PBS and samples analysed using a ViCell® Cell Viability Analyser (Beckman Coulter, High Wycombe, UK) for VCD and Viability. Cells were left for a further 24 hours to grow, and analysed for VCD and Viability again.

3.5.5.2. Transferral to shaking flask culture

Aliquots were taken from the T75 Flasks for seeding of Erlenmyer Flasks, pelleted by centrifugation at 180 *x g* for 5 minutes and resuspended in culture medium (Gibco® CD-OptiCHO with 8 mM Gibco® L-Glutamine (both ThermoFisher Scientific, Paisley, UK) and 75 nM Methotrexate (Sigma-Aldrich Ltd, Dorset, UK)). Cells from the transfected evolved cell line (DE) were seeded at 0.2 x10^6 cells mL^{-1}, 60 mL culture volume in E250 Erlenmyer Flasks with Vented Cap (Corning®, Tewksbury, US). Cells from the transfected host cell line (C2A) were seeded at 0.2 x 10^6 cells mL^{-1}, 30 mL culture volume in E120 Erlenmyer Flasks with Vented Cap (Corning®, Tewksbury, US).

Cells were incubated as described previously ("3.1.2 Maintenance and routine sub-culture"), being sub-cultured every 3-4 days, with culture volume being adjusted as necessary for experimentation.

3.6 Statistics

Unless stated otherwise, all experiments were conducted in triplicate. Statistical significance was calculated in GraphPad Prism software using the Holm-Sidak method student's t-test, where α = 5.000%. The decision to use a T-test to determine statistical significance was based on the fact that only two sets of data were compared at any one time (such as the comparison between the fluorescence intensity from CTL cells and cells treated with Ascorbic Acid, or the comparison between Day6 integral Viable Cell Density of 7C3 and 6A8), rather than comparing all data sets for a given variable (such as the Cell Specific Productivity of all conditions in Fed-Batch Experimentation Trial 1). In the latter case, ANOVA would have been much more powerful, and more appropriate to ensure reduction of error propagation. However, no wide-scale comparisons were conducted and so it was decided that a t-test would be sufficiently accurate.

Chapter Summary

All cell lines utilised in this study were DG44 (Urlaub *et al,* 1983) derived CHO cell lines developed in-house at FUJIFILM Diosynth Biotechnologies, Billingham, UK. Culture processes predominantly involved shaking flask batch or fed-batch culture in vented Erlenmyer flasks at varying volumes, although static incubation in multi-well plates was also conducted. Experimental activity included anti-oxidant supplementation of culture media, staining and fixation of cells using fluorescent dyes, use of flow cytometry analysis, directed evolution through chronic exposure to Hydrogen peroxide and transfection with circular and linearised DNA. Research was conducted on site at both Sheffield University and FUJIFILM Diosynth Biotechnologies, both UK.

Chapter 4:
Characterisation of Cell Lines

Contents

List of Abbreviations

CHO	Chinese Hamster Ovary
DHFR	Hihydrofolate Reductase
IVCD	Integral Viable Cell Density
$IVCD_{MAX}$	Maximum Integral Viable Cell Density
μ	Growth Rate
VCD	Viable Cell Density
q_P	Cell Specific Productivity
R&D	Research and Development
APC	Apollo Producer Cell Line
MFA	Metabolic Flux Analysis
HP	High Producing
LP	Low Producing
TCA Cycle	Tricarboxylic Acid Cycle
ΔL	Change in Lactate Concentration
ΔG	Change in Glucose Concentration
q_G	Cell Specific Glucose Consumption
q_L	Cell Specific Lactate Production
rMAb	Recombinant Monoclonal Antibody
RFU	Relative Fluorescence Units

List of Figures

4.1 Introduction

Since their initial isolation from an outbred Chinese Hamster by Dr. Theodore T. Puck in 1957, CHO cells in their various forms have been shared and adapted by laboratories around the world, meaning that when one refers to a "CHO cell" one could be referencing a cell type from a varied list of phenotypes, that if introduced individually today would certainly not be recognised as the "same" cell type. The popularity of CHO can be seen in even the early stages post description, with extensive use seen from the late 1950s through to the late 1970s; their large chromosomes and cell culture practicality were highly favoured for both molecular and classical cell genetics (Wurm, 2013). However, much as the products of those investigative efforts are used and exploited today both in academia and industry (such as the now genetically sequenced CHO-K1 (Xu *et al*, 2011), the CHO-S suspension adapted cell lines (Wurm, 2013), or the *DHFR*-negative CHO-DG44 cell lines (Derouazi *et al*, 2006)), there is little understanding of the similarities and differences between the origins and phenotypes of these largely disparate cell lines.

When cell lines were first distributed to laboratories, and in the subsequent work done with them, little attention was paid by researchers in noting the methods by which they attained their novel versions of these cells lines, or even in adequately (by today's standards) noting the culture conditions used in even routine subculture and maintenance (Wurm, 2013). Compounding this, any novel CHO cell line that may have been developed since then in an industrial setting is itself shrouded in secrecy, since competing companies are reluctant to reveal to their rivals their exact cloning and optimisation strategies. Indeed, it has been suggested that given the inherent (and favoured) volatile nature of CHO cells and the large cell numbers seen in manufacture scale ups, it is logical to infer that the cells being inoculated into the final 2000L bioreactor constitute an entirely separate quasispecies to those that were first brought out of cryo-preservation several weeks previous, if we use this term in the sense of the seminary papers published by Eigen and Schuster in the late 1970's. Eigen and Schuster coined the term "quasispecies" to describe the environment whereby a large "cloud" of offspring exists, leading to the expectation of a large fraction of said offspring carrying a mutation (Eigen & Schuster, 1977-78). Regulatory approval requires proof of stability though stable biomanufacturing phenotype and repeated rounds of cloning, but there is no current requirement for proof of genetic stability from cryo-revival to full production scale cultures.

The need to ensure increasing pushes towards collating large-scale 'omics data for CHO cell lines produces relevant and useable information is not one that is lost on the biomanufacturing community, with work being carried out to establish the genetic correlation between cell lines and the Chinese Hamster itself (Lewis *et al*, 2013). There is increasing evidence that it should perhaps not even be accurate to describe a single flask of CHO cell culture as homogenous. To use genetics as an

example, studies have shown that a CHO-K1 SV cell line grown under protein free conditions (as is current standard in both industrial and academic work) shows an extremely broad distribution of chromosome number of 10-30 chromosomes (Hazelwood, 2006). In this work conducted during a PhD investigation under the guidance of Prof. Alan Dickson, 10%, 13%, 17%, 7% and 12% of the cell culture showed 16, 17, 18, 19 or 20 chromosomes, respectively, and further studies of subclonal populations of this parental cell line indicated even more varying karyotypes with no one subclone even vaguely matching the karyotypic distribution of their progenitors (Hazelwood, 2006). Such variation in each cell culture population means that in instances of cloning it is not clear what chromosome number has been cloned out, and there has been no investigation as to whether chromosome number affects cloning efficiency. In addition, frequently used interferences such as transfection procedures or selection stressors have been shown to further (and dramatically) introduce further genomic restructuring (Landry *et al*, 2013).

Given that the nature of the CHO quasispecies can be further altered by such things as culture conditions, culture media and additives and cryopreservation techniques (to name a few), it is necessary for researchers to establish the specific phenotype of their cell line prior to experimentation, in order to have an appropriate set of parameters with which to compare their results. Any comparison with other cell lines, even those apparently close in lineage to the one being tested, could very well be completely irrelevant due to the variance described above. Thus, this following chapter will aim to describe the data obtained when characterising the various cell lines used in this study. Although all closely related (see Fig 4.1), it was necessary to establish their respective growth, productivity, redox and metabolic profiles.

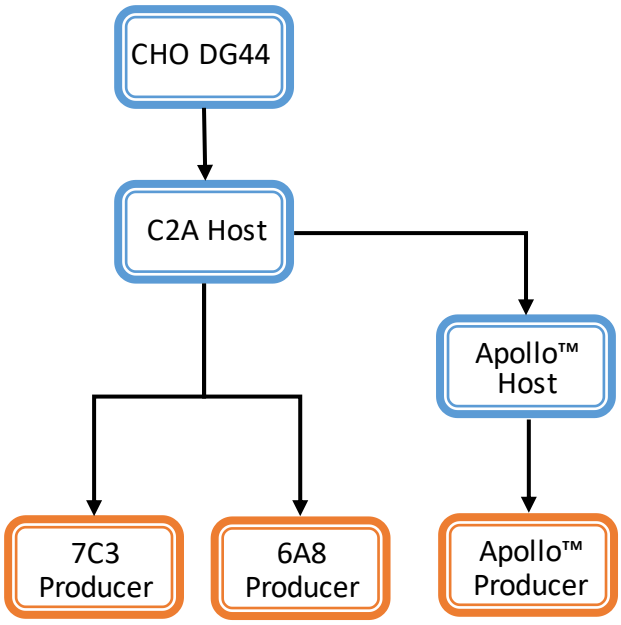

Figure 4.1. Lineage of Cell Lines used in this investigation, in relation to each other.

4.2 Experimentation

Host and Producer cell lines were cultured according to Chapter 3 "*Materials and Methods*" sections 3.2.2, 3.2.3 and 3.2.4; "*Maintenance and Routine Sub-culture*", "*Cryopreservation*", "*Cell Revival*", pgs. 34, 35, 36, respectively. Producer cell lines under fed-batch culture processes were subject to the feeding regime as detailed in Chapter 3 "*Materials and Methods*" section 3.2.2 "*Maintenance and Routine Sub-culture*", pg34. Unless otherwise stated, cells were cultured in triplicate at 30 mL culture volume in 125 mL Vented Erlenmyer Flasks at 140 rpm, 36.5 °C and 5 % CO_2. Viable Cell Density and % Viability of cultures was determined using a Vi-CELL Cell Viability Analyzer, as described in 3.2.2 "*Maintenance and Routine Sub-culture*", pg34. Integral Viable Cell Density (IVCD) and growth rate (μ) were calculated according to Equation 3.1 and Equation 3.2, respectively (pgs. 34 & 35).

Glucose and Lactate consumption and production of host cell line C2A and producer cell lines 7C3 and 6A8 was established and analysed according to 3.2.7 "*Glucose and Lactate Determination: Cedex Bioanalyser*", pg38.

Productivity of producer cell lines was determined according to 3.2.5 "*Productivity*", pg36. Reactive Oxygen Species content of producer cell lines was determined using CellROX staining and flow cytometry analysis. For batch cultures of 7C3 and 6A8, this was conducted according to 3.2.6 "*Reactive Oxygen Species Quantification*", pg37. For fed-batch cultures of APC, 7C3 and 6A8, this was conducted according to 3.4.3 "*Reactive Oxygen Species Quantification*", pg43.

4.3 Growth Profiles

4.3.1 Batch Culture

At the beginning of the study, batch culture growth curves were performed for host cell line C2A and producer cell lines 7C3 and 6A8. Whilst industrially, fed-batch processes have long been favoured over batch, the shorter culture process provides useful information on baseline phenotypic properties of a cell line without needing to invest as much time into the experimentation. Indeed, host cell lines would never be subject to a fed-batch process in industry as it would be a waste of resources to maintain a cell line for up to two weeks when it is not producing anything; however when intending to develop novel cell lines using the original host, the researcher finds value in seeing the growth profile of their cell line past the first 3 days (the time allowed between sub-cultures).

In batch culture, C2A grew to a peak Viable Cell Density (VCD) of 3.16 x 10^6 cells/mL at 96h of culture (Fig. 4.2.B), with culture period lasting for 120 h before culture was stopped, with Viability having been recorded below 70 % at this point (Fig 4.2.D). Maximum Integral Viable Cell Density

($IVCD_{MAX}$) for the cell line was therefore calculated to be 201 cell•h/mL at 120 h (Fig. 4.2.F). This represents a relatively slow and short period of growth for CHO cell lines compared to the industry standard, however this impaired growth is likely due to these cells being DG44 derived and the *DHFR* deficiency associated with that lineage. When first developed, it was thought that media supplementation with hypoxanthine and thymidine (HT) was sufficient to propagate *DHFR*- cell lines derived from DG44 or DUXB-11. However work by Florin *et* al (2011) demonstrated that the lack of DHFR gene was the cause of the dramatically decreased cell density in such cell lines, and only re-introduction of said gene can restore the high densities required in industry. The effect of DHFR reintroduction was dose-dependent and could be used in lieu of the standard (and time consuming) MTX amplification normally utilised in the development of these cell lines (Florin *et al*, 2011). When producer cell lines were developed from C2A through generation of stable pools, the *DHFR* gene was co-transfected with the product gene, and indeed we see improved growth profiles in the two producer cell lines used from these stable pools: 7C3 and 6A8.

In a batch process, 7C3 grew to a peak VCD of 7.87 x 10^6 cells/mL and 6A8 grew to a peak of 8.72 x 10^6 cells/mL (Fig. 4.2.A), with cell culture lasting 192 h, at which point Viability was measured below 70 % (Fig. 4.2.C). Thus, IVCD at 120 h for 7C3 and 6A8 was calculated at 700.6 cell•h/mL and 677.4 cell•h/mL, respectively (Fig. 4.2.E). The growth profiles of these two producers closely correlate, and do not show any significant differences in terms of IVCD, growth rate (μ) or Viability throughout the course of a 192 h culture period. All batch culture work relating to producer cell lines was conducted in the laboratories in the University of Sheffield. There was no need to produce growth curves for the Apollo™ Host or Apollo™ Producer in a shaking flask batch process. The Apollo™ Host was only used in plate based, transient transfection experiments and the Apollo™ Producer only used in shaking flask fed-batch experimentation, the growth profiles of which will follow in subsequent sections of this chapter.

4.3.2 Fed-Batch Culture

Perfusion or continuous culture and fed-batch culture are the two dominant production processes for large-scale biologics production (Meuwly *et al*, 2006). It has been shown that continuous control of glutamine and glucose levels can reduce build-up of toxic waste-products such as lactate and ammonia, and cells could be effectively grown for weeks at a time, thus optimising product yield (Meuwly *et al*, 2006). However, perfusion processes can be complicated to set up and maintain at high volumes, and ultimately such processes are often deemed not to be financially viable for industrial biologic production (Meuwly *et al*, 2006). Fed-batch cultures, however, are much simpler to operate and scale up, and far cheaper to optimise, whilst providing extended culture time and

thus product yield in comparison to batch cultures (Bibila & Robinson, 1995). Fed-batch processes have, therefore, long been the culture mode of choice in industry, and thus knowledge of cell line growth profiles in such conditions is pertinent. Optimisation of a feeding strategy is as time-consuming and laborious as optimisation of basal media. However, Research and Development (R&D) departments of biologics manufacturing companies will often have favoured media and feeding strategies suitable for their own cell lines that are employed unless there is a specific need for extra optimisation. In this case, the same feeding strategy was used for all three cell lines in order to minimise the variables in experimentation.

Fed-batch culture growth curves were performed for all three producer cell lines: 7C3, 6A8 and Apollo™ (APC). VCD for 7C3 and APC peaked at 168 h at 8.89 x 10^6 cells/mL and 9.5 x 10^6 cells/mL, respectively. 6A8 responded better to the selected feeding strategy, and VCD for this cell line peaked at 240 h, reaching 13.02 x 10^6 cells/mL at this timepoint (Fig. 4.3.A). Culture period for 7C3 and APC fed-batch runs lasted 264 h, and for 6A8 288 h, with $IVCD_{MAX}$ thus being calculated as 727 cell•h/mL, 1210 cell•h/mL and 822 cell•h/mL for 7C3, 6A8 and APC, respectively (Fig. 4.3.C). It is useful to note that for the first 168 h of culture the growth rates of the three cell lines correlate closely, showing little to no variation. However, readings taken at the 240 h time-point show that whilst 7C3 and APC have slipped into negative growth (also known as the death phase of a culture period), 6A8 growth remains positive, and indeed is slightly increased (Fig 4.3.D). 6A8 does enter the death phase after this point, and this extended growth explains why this cell line was able to reach a much higher $IVCD_{MAX}$ than the other two. Feed was added to all three cultures at 168 h (after the ViCell samples were taken for growth analysis), and it is curious that this feed boosted growth for 6A8 but seemingly not for the other two.

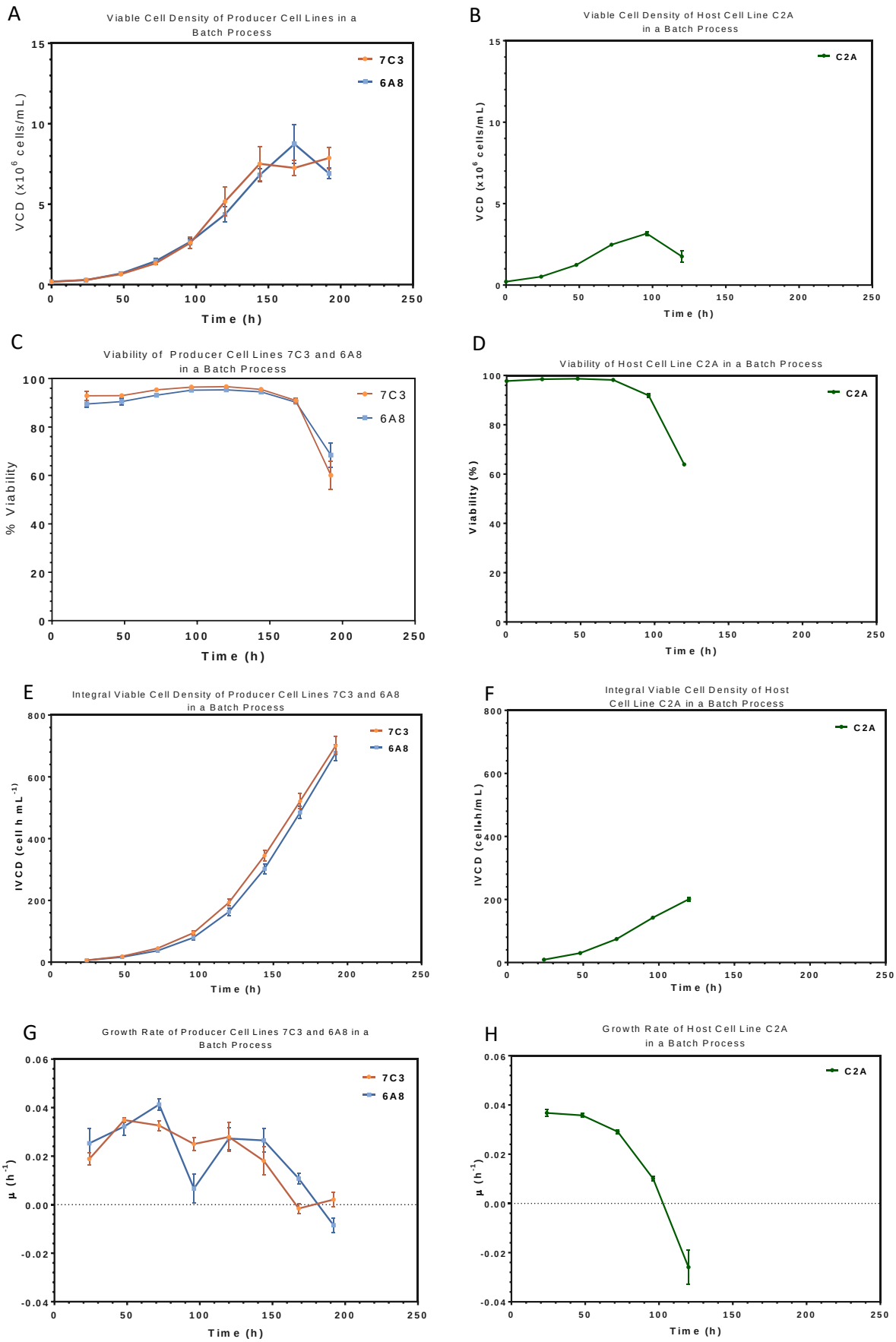

Figure 4.2. In all cases, cells were cultured in triplicate E125 flasks at a culture volume of 30mL each at 140rpm, 37°C and 5% CO_2. Data points indicate the mean average of measurements taken from said triplicates at the indicated timepoint, with error bars indicating the Standard Error of the Mean, as calculated by GraphPad Prism software. Graphs A, C, E and G indicate VCD, Viability, IVCD and growth rate (μ), respectively, for 7C3 and 6A8 in a batch process. Graphs B, D, F and H indicate VCD, Viability, IVCD and μ, respectively, for C2A in a batch process.

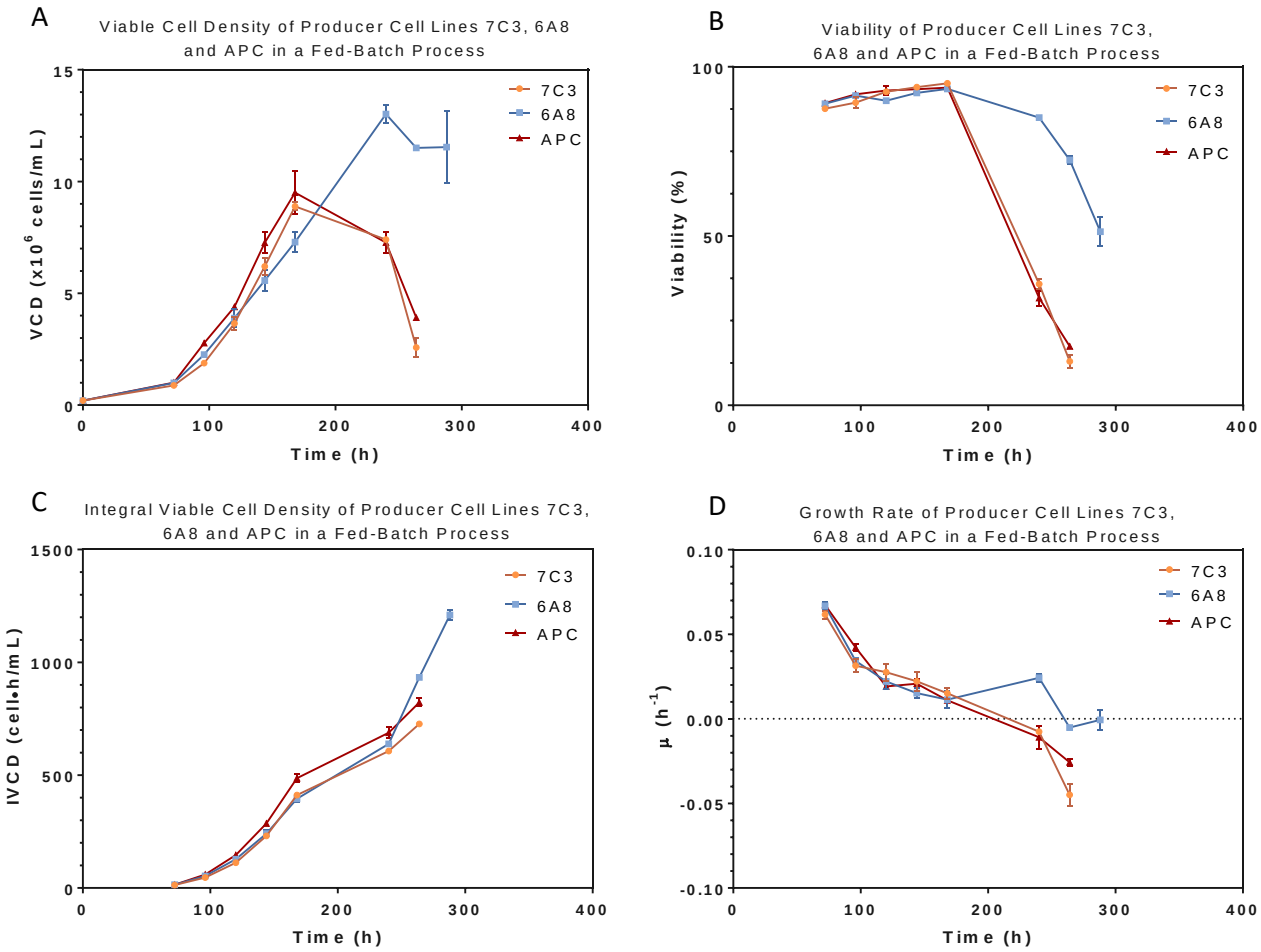

Figure 4.3. In all cases, cells were cultured in triplicate E125 flasks at a culture volume of 30 mL each at 140 rpm, 36.5 °C, 5 % CO_2 and 80 % humidity. Data points indicate the mean average or measurements taken from said triplicates at the indicated timepoint, with error bars indicating the Standard Error of the Mean, as calculated by GraphPad Prism software. A: VCD of 6A8, 7C3 and APC over a fed-batch culture period, B: Viability of 6A8, 7C3 and APC over a fed-batch culture period, C: IVCD of 6A8, 7C3 and APC over a fed-batch culture period, D: Growth rate (μ) of 6A8, 7C3 and APC over a fed-batch culture period.

B

A

4.4 Metabolic Profiles

Typically, cultured CHO cells used in biopharmaceutical production display a biphasic metabolic profile whereby at late exponential to mid stationary phase, the cells undergo a metabolic 'switch' from lactate production to lactate consumption. The Warburg metabolism seen in the first phase of cell culture is characterised by an increased reliance on aerobic glycolysis for energy production and oxidation of glutamine as a carbon source, leading to significantly increased production of waste metabolites lactate and ammonia (Zagari et al, 2013; Mulukutla et al 2010; Zhou et al, 2011). When striving to maximise biologics production, this energetically unfavourable metabolism raises two main concerns; firstly, that lactate and ammonia are toxic to the cell and thus likely to impact on growth and productivity (Zagari et al, 2013), and secondly that whilst growth rate is seen to increase, protein production in this period is much lower, suggesting that should metabolic differences occur at other stages in culture they may have a negative impact on product yield.

Templeton et al (2013) conducted Metabolic Flux Analysis (MFA) of high producing (HP) and low producing (LP) CHO cell lines and, concurrent with a complementary metabolic study by Dean & Reddy (2012), established that biomass accumulation switched from the largest outgoing flux in exponential phase to being negligible in the decline phase of growth – a logical conclusion given that in the later phases of culture cells have essentially stopped growing. Additionally, they observed that lactate production reduced significantly over the course of the exponential phase, and then reversed direction to consumption during stationary phase. This analysis is consistent with previous observations made of the glucose and lactate metabolism in CHO cell culture, and is reflected in their further observations that the overall Tricarboxylic Acid Cycle (TCA cycle) flux is much higher during the later period of culture than in the earlier stages (Dean & Reddy, 2012; Templeton et al, 2012).

It is clear, then, that metabolism plays a critical role in the performance and productivity of cell lines selected to produce biologics, and so an understanding of the specifics of that cell line's metabolism could be advantageous. It can be seen from supernatant analysis using a Cedex Bioanalyser that prior to stable pool generation, the cell line C2A shows no metabolic switch and continues to produce lactate whilst consuming glucose right to the end of the 6 day culture. Final lactate concentration peaked at 2071.25 mg/L 150 h into culture (from a starting level of 152.67 mg/L at 2 hours) and final glucose concentration stood at 1271.7 mg/L, down from 3002.54 mg/L (Fig. 4.4.A). When observing the supernatant glucose and lactate content of fed-batch producer cells 7C3 and 6A8, derived from stable pools generated using C2A, the 'switch' discussed earlier can now be observed. Lactate content increases in the first 144 h of culture, reaching 1312.29 mg/L and 1302.83 mg/L (7C3 and 6A8, respectively) before the supernatant concentration is seen to drop off fairly steeply. Lactate concentration in the supernatant continues to drop, and by the end of culture

(240 h) has returned to a level only slightly higher than that at the beginning of culture: 7C3 shows a concentration of 436.83 mg/L, up from 104.09 mg/L at 24 h; 6A8 shows a concentration of 441.34 mg/L, up from 129.26 mg/L at 24h (Fig. 4.4.B).

When comparing the rates of lactate production (Rate of ΔL) and glucose consumption (rate of ΔG) based purely on changes in supernatant concentration, it is observed that the overall rate of glucose consumption in both 7C3 and 6A8 is reasonably static in the exponential stage of culture growth, increases at late exponential and continues to increase throughout culture before plateauing again in death phase (Figure 4.5.A). Overall lactate production, in contrast, starts high during exponential growth and decreases steadily, before consumption starts at 144 h. However, once lactate consumption begins, rate of consumption quickly becomes static (Fig. 4.5.B), and then decreases in death stage. Rates of ΔG and ΔL were calculated according to Equations 3.6 and 3.7 (3.2.7. "*Glucose and Lactate Determination*", pg38).

More pertinent than the isolated rates of glucose consumption and lactate production/consumption, however, are the cell specific rates; that is, the correlation of the rate of concentration change with the growth of the cells. Cell specific glucose consumption (q_G) and lactate production (q_L) were calculated according to Equations 3.8 and 3.9 (3.2.7. "*Glucose and Lactate Determination*", pg38). It can now clearly be seen that in the cases of the two producer cell lines, q_G is much higher in the early exponential phase of growth (48 to 72 h), and slows down to a plateau by the time cultures reach stationary phase, at around 96 to 120 h (Figure 4.6.A). This now brings into line the metabolic profiles of these producer cell lines with the Warburg metabolism previously described in exponentially growing CHO cell cultures (Mulukulta *et al*, 2010). Similarly, q_L for both producer cell lines, as well as the host cell line, is much higher at 48 h, dropping steeply over the next 3 days of culture. Whilst production rate becomes negative at 144 to 168 h of culture in the producer cells lines, and not at all in the host cell line, It should be noted that there is no significant difference between q_L of C2A and that of the two producer cell lines for the duration of the C2A batch culture, suggesting that the switch to lactate consumption is merely a factor of duration of culture, and does not indicate a dramatic metabolic shift after the stable pool generation process. The constant rate of consumption of glucose from 144 h onwards despite the onset of lactate consumption correlates with the findings of Templeton *et al* (2013), who saw no direct impact on stationary phase glucose consumption when lactate was being consumed. The data thus indicates that the producer cell lines derived from host cell line C2A exhibit typical production CHO cell metabolic characteristics; Warburg metabolism characterised by high glucose consumption and high lactate production in early exponential phase is followed by a switch to lactate consumption and a decrease in the rate of glucose consumption in late exponential and stationary growth phase.

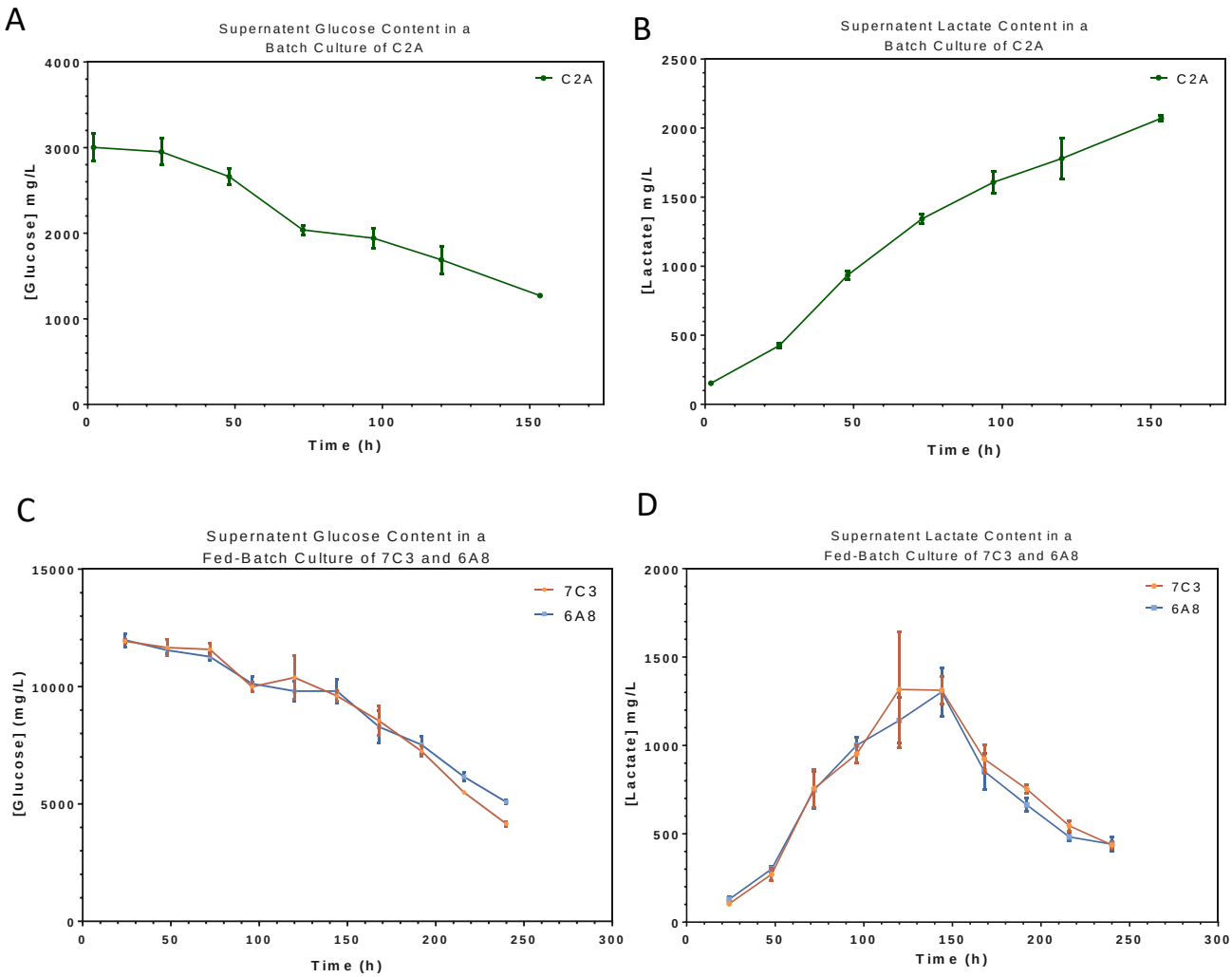

Figure 4.4. Concentration of Glucose and Lactate in supernatant samples taken from A) Batch culture of C2A cell line and B) Fed-batch culture of 7C3 and 6A8 cell lines. Samples were taken from triplicate flasks and data points indicate the mean average of these three samples, error bars indicate Standard Error of the Mean. Samples were analysed for glucose and lactate content using a Cedex Bioanalyzer.

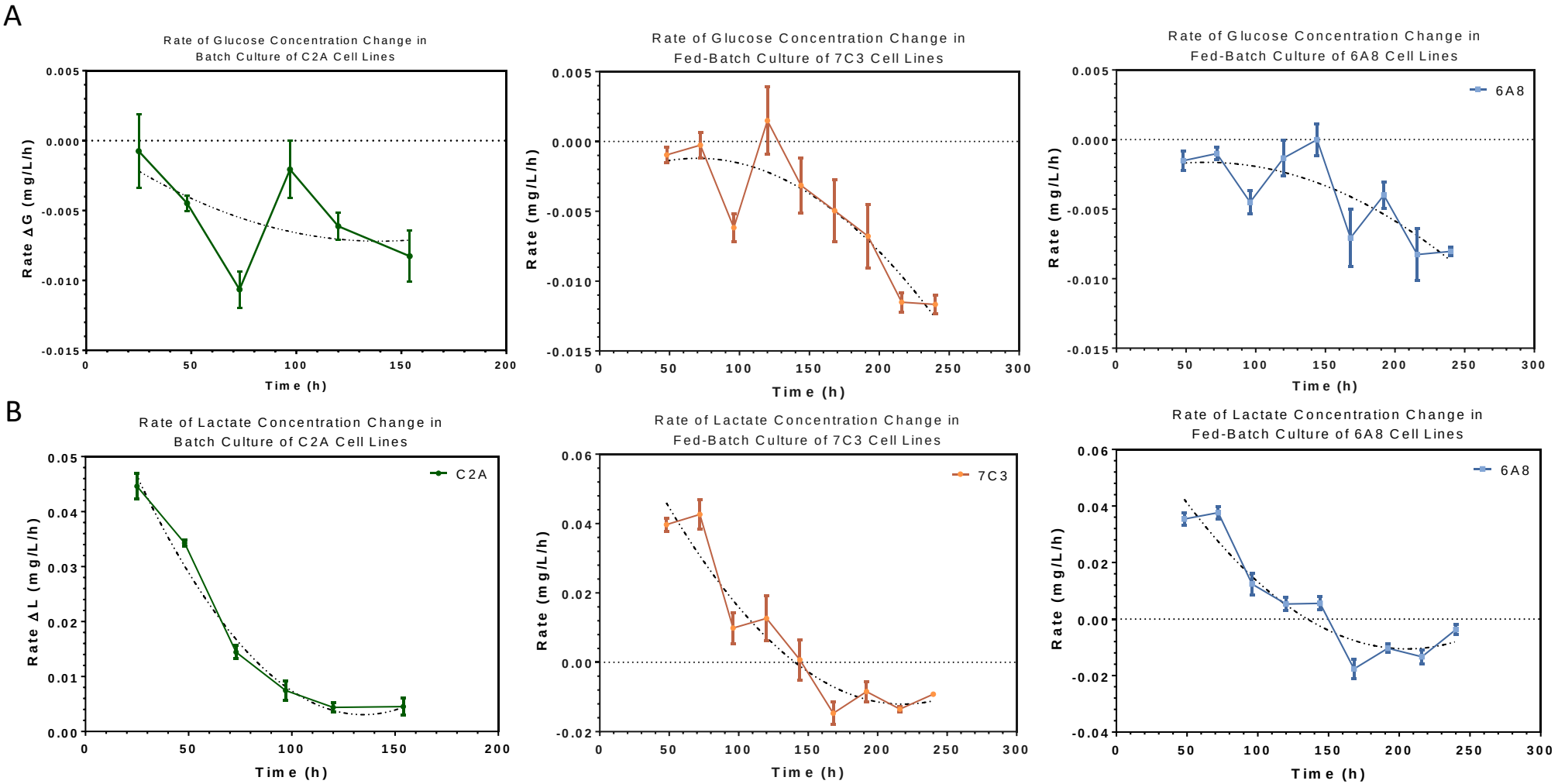

Figure 4.5. Rates of A) Glucose consumption and B) Lactate production as indicated by a change in concentration during batch culture of C2A and fed-batch culture of 7C3 and 6A8. Data points are the mean average of triplicate samples with error bars indicating Standard Error of the Mean. A). Lines of best fit have R^2 values of 0.183, 0.6059 and 0.4731 for C2A, 7C3 and 6A8, respectively. C2A consumption rate is significantly higher than the other two cell lines at 48 h, 72 h and 120 h, $p \leq 0.05$. 6A8 consumption rate is significantly lower than 7C3 at 240 h, $p \leq 0.1$. B) Lines of best fit have R^2 values of 0.9642, 0.8474 and 0.858 for C2A, 7C3 and 6A8, respectively. There are no significant differences shown in the rate of lactate production between the three cell lines.

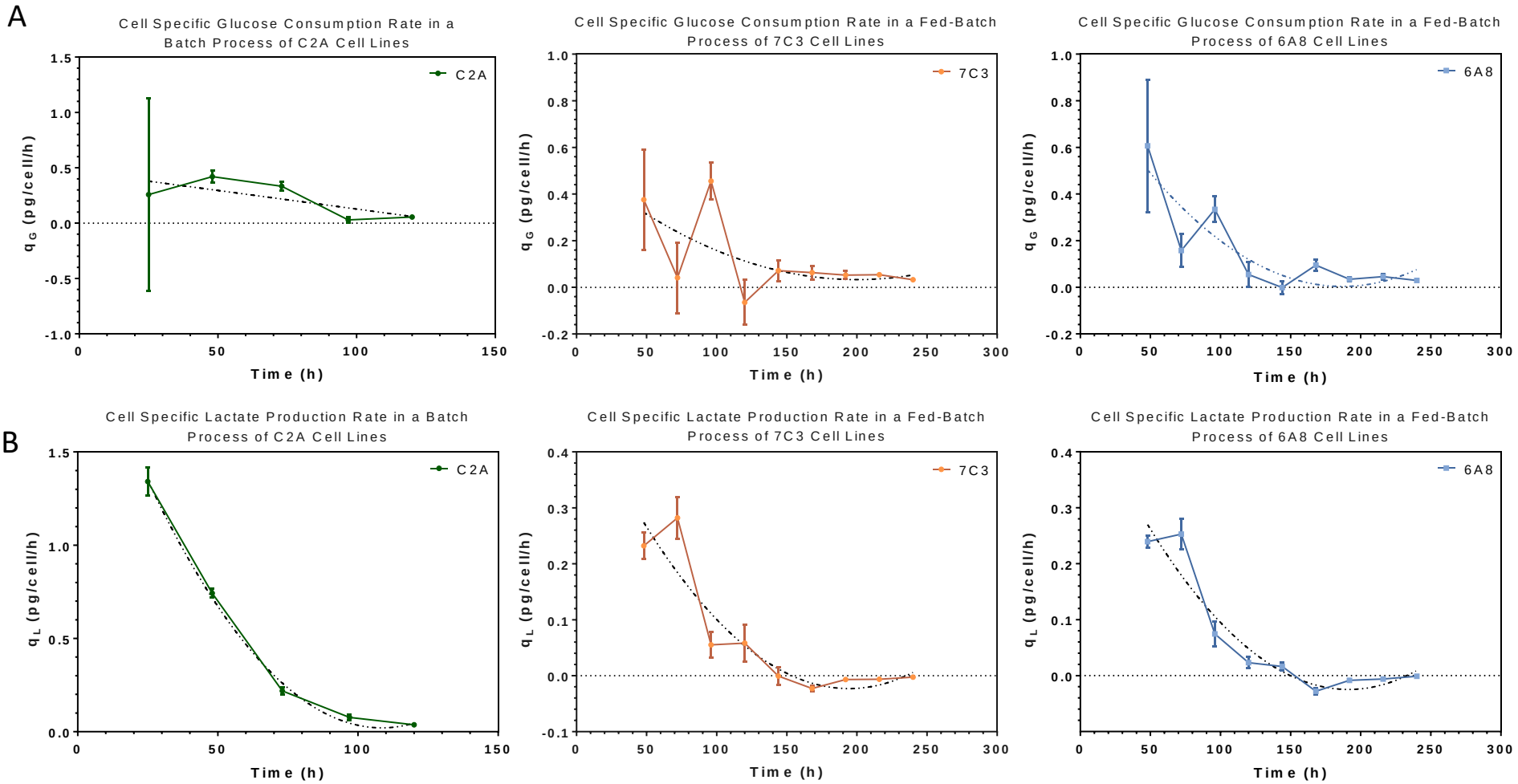

Figure 4.6. Cell Specific Rates of A) Glucose consumption and, B) Lactate production, as calculated by a change in concentration as a factor of IVCD during batch culture of C2A and fed-batch culture of 7C3 and 6A8. Data points are the mean average of triplicate samples with error bars indicating Standard Error of the Mean. A). Lines of best fit for C2A, 7C3 and 6A8 have R^2 values of 0.039, 0.20 and 0.48, respectively. C2A is significantly higher than 7C3 at 96 h only, $p \leq 0.05$ and significantly higher than 6A8 at 96 h only, $p \leq 0.1$. Rates between the two producer cell lines show no significant difference. B) Lines of best fit for C2A, 7C3 and 6A8 have R^2 values of 0.99, 0.79 and 0.88, respectively. C2A is significantly higher than 7C3 and 6A8 at 48 h only, $p \leq 0.001$. Rates between the two producer cell lines show no significant difference.

4.5 Productivity

The ultimate goal of any cell engineering endeavour in the biologics production industry is to increase overall product titre and productivity rate. Productivity profiles of all three producer cell lines in this study were therefore established, in order to accurately assess whether engineering efforts had a beneficial or detrimental effect. All three producer cell lines are producing the same recombinant monoclonal antibody (rMAb) product.

4.5.1 Batch Culture.

Batch processes are not generally favoured in industrial production processes as the resultant titres are comparably low when compared to fed-batch processes, which enable longer productive culture periods and thus higher titres. However, in a research context, batch cultures can provide a relatively rapid indication of the effects of perturbations to the system without having to commit up to two weeks' worth of time for each untested condition. In initial trials of anti-oxidant media supplementation conducted at the University of Sheffield site, 7C3 and 6A8 were cultured for 192 h (8 days) and supernatant samples taken, to be analysed using the Octet® HTX System (PALL ForteBio, Southampton, UK) (3.2.5.2 "*IgG Quantification: Octet*", pg36). During experimentation, a novel protein detection assay was developed in house (ValitaTiter; Thompson *et al*, 2016), and so batch production profiles were also generated for this assay.

Despite the growth profiles of the two producer cell lines being almost identical (Fig. 4.7.A and B), the Octet generated production profiles are very different (Fig.4.7.C). 7C3 reached a maximum titre of 138.8 µg/mL at 168 h, whereas 6A8 achieved nearly double this a day earlier, with a maximum observed titre of 262.7 µg/mL at 144 h. A similarly large difference is also observable when using ValitaTitre, with 7C3 achieving a maximum titre of 221.87 µg/mL and 6A8 achieving maximum titre of 355.2 µg/mL.

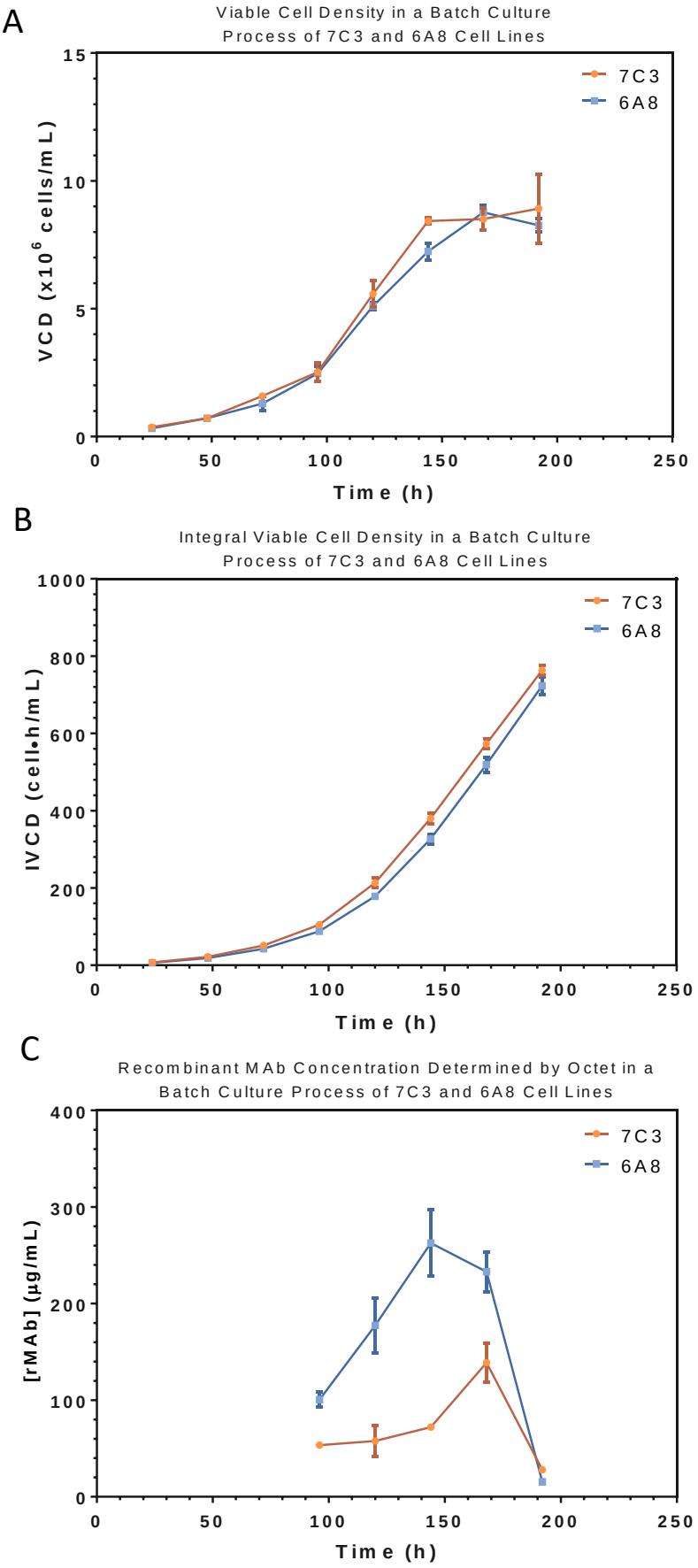

Figure 4.7 Growth and Productivity of 7C3 and 6A8 Cell Lines in a Batch Process. (A) Viable Cell Density of 7C3 and 6A8 Cell Lines and (B) Calculated Integral Viable Cell Density of the same cell lines. (C) rMAb Titre of 7C3 and 6A8 as determined by the Octet. Data points indicate the Mean average of triplicate cell cultures, and error bars indicate Standard Error of the Mean.

4.5.2 Fed-Batch Culture

Cell Lines 7C3 and 6A8 were cultured in a fed-batch process as described in section 3.2.2.1 "*Fed-batch Feeding Strategy: Sheffield*", pg35. In a continuation from the observed productivity in a batch process, production of the recombinant MAb was much higher in 6A8 cultures than in 7C3. A through-culture analysis of productivity was analysed using the Octet and is displayed in Fig.4.8. rMAb concentration in 7C3 cultures at 240 h was 284.8 µg/mL and had not appeared to peak before the culture died, whereas 6A8 achieved a titre of 348.8 µg/mL at 216 h and production appeared to have slowed.

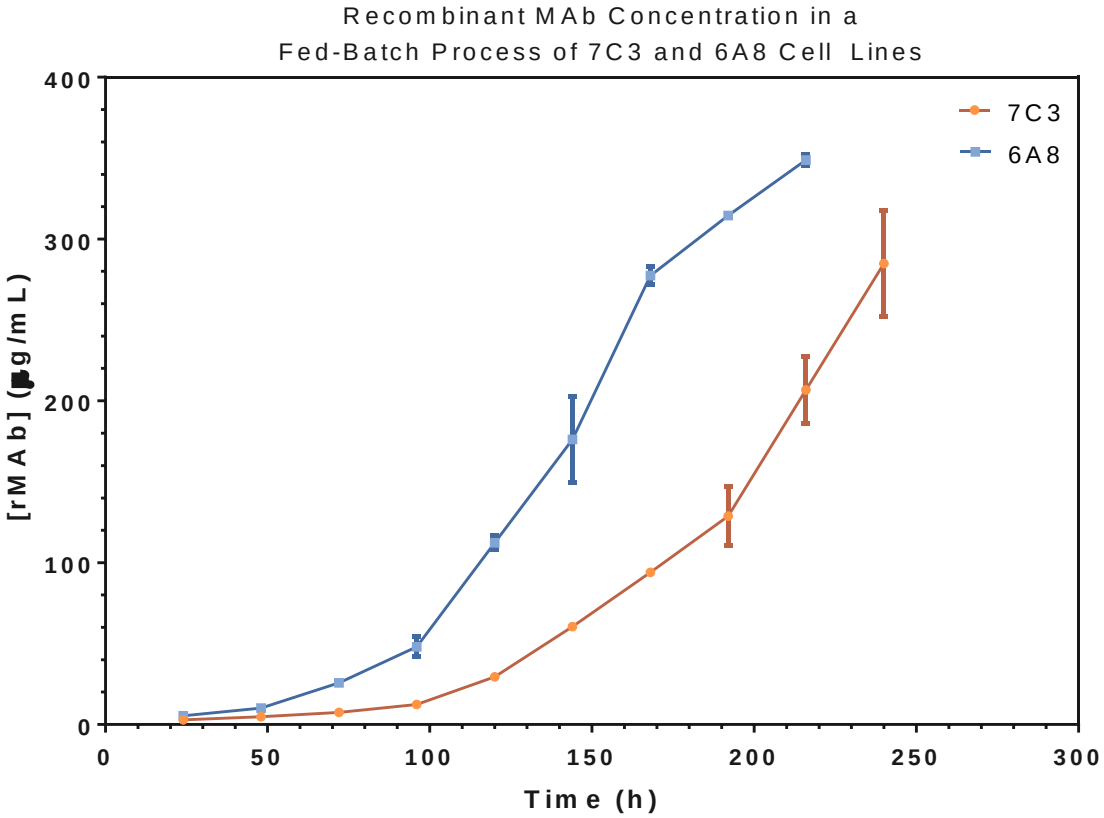

Figure 4.8. Concentration of rMAb in supernatant samples taken from triplicate 7C3 and 6A8 cultures, as determined by analysis on the Octet. Data points indicate the Mean average of triplicate cell cultures, and error bars indicate Standard Error of the Mean. From 96 h onwards, data points for 6A8 are significantly higher than 7C3, $p \leq 0.05$.

All further fed-batch experimentation was conducted at the FUJIFILM site, and so a comparison between 7C3, 6A8 and APC was necessary. Feeding strategies employed in this case were different to those employed previously, as detailed in 3.2.2.2. "*Fed-batch Feeding Strategy: FUJIFILM Diosynth Biotechnologies*", pg35. Figure 4.9 indicates the rMAb concentration from fed-batch cultures at 3 timepoints in the culture period. "Early" indicates samples taken at 120 h, "Mid" indicates samples taken at 168 h and "Late" indicates samples taken at 288 h. For product concentration

determination in fed-batch experimentation from this point, the ValitaTITER assay was used (3.2.5.3 "*IgG Quantification: Fluorescence Polarisation*", pg37).

At 288 h, supernatant rMAb concentration from 7C3 cultures stood at 295.52 µg/mL, from 6A8 cultures rMAb concentration was 769.39 µg/mL and from APC cultures rMAb concentration was 1951.1 µg/mL. Despite being maintained in culture 48 hours longer in this case, the final titre for 7C3 is not significantly different to that obtained in the University of Sheffield fed-batch run. On the other hand, the titre for 6A8 in the FUJIFILM fed-batch run was more than double that of the previous experimentation. There had been no previous experimentation with the APC cell line and so there can be no comparison between feeding strategies. The more recently developed APC cell line is, however, clearly a much better producer of this easy to express rMAb than the other two, with a final day titre of 1951.1 µg/mL being roughly 6-fold higher than that of 7C3, and 2.5-fold higher than 6A8 (Fig. 4.9.A). During the "Early" stage of production, titres from 6A8 and APC cultures are not significantly different, but by the "Mid" stage of culture titre from APC is significantly higher than that of 6A8 and 7C3 ($p>0.01$), with the trend for increased titre continuing through to the end of culture. These differing rMAb profiles indicate that the production phase for APC is much more efficient later on in culture, enabling the much higher production profiles seen in the mid and late stages.

Comparison of cell-specific productivity further confirms the conclusion that late stage production in APC is far superior to that of the other two cell lines (Fig. 4.9.B). In both the "Early" and "Mid" stages of culture, there is no difference between APC and 6A8 productivity. However, APC specific productivity on the final day of culture is 4.5-fold higher than 6A8, and in both "Mid" and "Late" stages specific productivity of APC is 10.4-fold and 5.7-fold higher than that for 7C3, respectively.

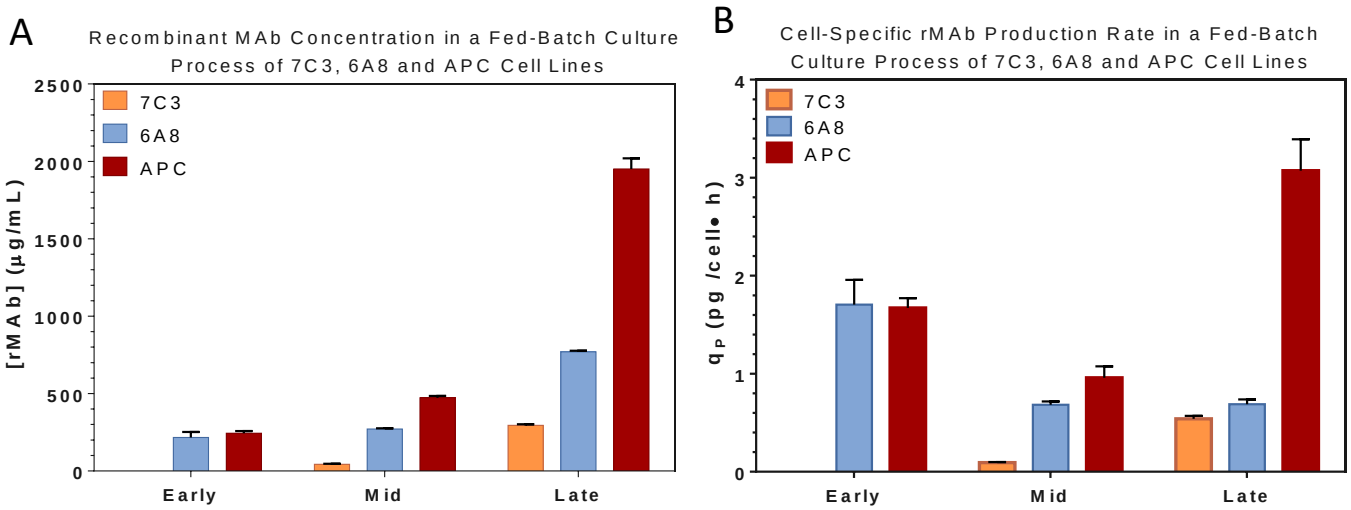

Figure 4.9 Productivity of producer cell lines in a fed-batch process at 120h (Early), 168 (Mid) and 288h (Late) of culture. (A) rMAb titre µg/mL as measured by ValitaTITER assay. (B) Cell specific rMAb productivity pg/cell•h as determined with [rMAb] and IVCD according to Equation 3.5. All data points indicate Mean average of samples from triplicate cell cultures and error bars indicate Standard Error of the Mean.

4.6 Reactive Oxygen Species Content

Throughout the duration of this work, relative resistance to oxidative stress is to be determined through analysis of intracellular Reactive Oxygen Species (ROS). Intracellular ROS content was determined by staining cell samples with CellROX® Deep Red Reagent (Thermofisher Scientific, Paisley, UK) followed by analysis using flow cytometry. CellROX Deep Red Reagent is a cell-permeable fluorescent dye that has an excitation/emission spectra of ≈644/665nm, and has been widely used in the determination of oxidative stress in mammalian cells since its development (Weiss & Ito, 2015; Eshraghi *et al*, 2015; Garcia-Jérez *et al*, 2015 are some of the most recent publications listing its use). Upon oxidation by reactive oxygen species, CellROX Deep Red fluoresces with emission maxima of 665 nm. This specific cell-permeable dye developed by Thermofisher Scientific was selected over other dyes used for oxidative stress determination due to its retention post-fixation.

For all flow cytometry analysis, samples from untreated control cultures were analysed due to the potential for emission fluctuation in each experiment, however it is useful to compare the CellROX profile of each cell line to gauge the relative differences in oxidative stress resistance between the cell lines, and in different culture processes. Cell lines 7C3 and 6A8 were assessed for ROS content in batch and fed-batch processes, and APC was assessed for ROS content in a fed-batch process only. All values shown indicate the median average fluorescence at 620 nm of 10,000 events (cells) analysed using flow cytometry, after gating for viability.

4.6.1 Reactive Oxygen Species Levels in a Batch Process

Cell lines 7C3 and 6A8 were cultured in triplicate flasks in a batch process, as described in 3.2.2 "*Maintenance and routine sub-culture*" pg34. Cell samples were acquired, stained and fixed as described in 3.2.6.1 "*Cell Staining and Fixing*", pg37, and then analysed using an Attune® Autosampler flow cytometer (ThermoFisher Scientific, Paisley, UK) as described in 3.2.6.2 "Imaging using Flow Cytometry", pg38.

The ROS levels in 7C3 and 6A8 as determined by relative fluorescence at 72 h ("Early"), 120 h ("Mid") and 168 h ("Late") of culture are detailed in Fig. 4.10. It can be seen that 7C3 shows a slightly lower level of ROS in the Mid stage of culture (i.e. stationary phase) with a median fluorescence of 1352 Relative Fluorescence Units (RFU) versus 1772 RFU, and a p value of less than 0.05. However, in the Early and Late stages (i.e. growth and late stationary/death phases) there was no difference between the cell lines. There is a clear increase in ROS levels as culture time period progresses, with 7C3 showing an increase from 1008 RFU at 72 h to 2090 RFU at 168 h and 6A8 showing an increase from 1149 RFU to 2360 RFU at the same time points. This gradual increase in ROS levels correlates

with the knowledge that through the duration of cell culture, ROS production due to a variety of cellular processes eventually overwhelms the intrinsic cellular defence systems, leading to an increase in intracellular ROS levels.

4.6.2 Reactive Oxygen Species Levels in a Fed-Batch Process

Cell lines 7C3, 6A8 and APC were cultured in triplicate flasks in a fed-batch process, as described in 3.2.2.2 "*Fed-batch Feeding Strategy: FUJIFILM Diosynth Biotechnologies*", pg35. Cell samples were acquired, stained and fixed as described in 3.4.3.1 "*Cell Staining and Fixing*", pg43, at 144 h (Mid) and 264 h (Late) of culture and were then analysed using a FACSAria II Flow Cytometer (BD Biosciences, Plymouth, UK) as described in 3.4.3.2 "*Imaging Using Flow Cytometry*", pg44.

The ROS levels in 7C3, 6A8 and APC as determined by relative fluorescence are detailed in Fig. 4.11. At 144 h ("Mid" stage of culture, or stationary phase), there is no difference between 7C3 and 6A8 and only a slight difference between the two cell lines at 264 h ("Late" stage of culture, or death phase), with fluorescence intensity values of 7373 RFU and 6321 RFU, respectively, a statistically significant difference with calculated p value ≤ 0.1. The APC cell line, however, shows far less ROS content than the other two cell lines at both time points, indicating either that this cell line produces far less ROS than the other two, or that the intrinsic antioxidant defences in APC are stronger and thus the cell line is inherently more resistant to oxidative stress. As in the batch culture process, there is an increase in ROS levels between 144 h and 264 h, although the increase is smaller. There also appears to be an increase in APC ROS levels, although this increase is small and further experimental runs would be required to elucidate whether this result is true. Conversely to the other two cell lines, there appears to be a slight drop in 6A8 ROS levels in the latter stages of culture, although this is not supported by statistical analysis and further experimental runs would be required to elucidate whether this is a true result.

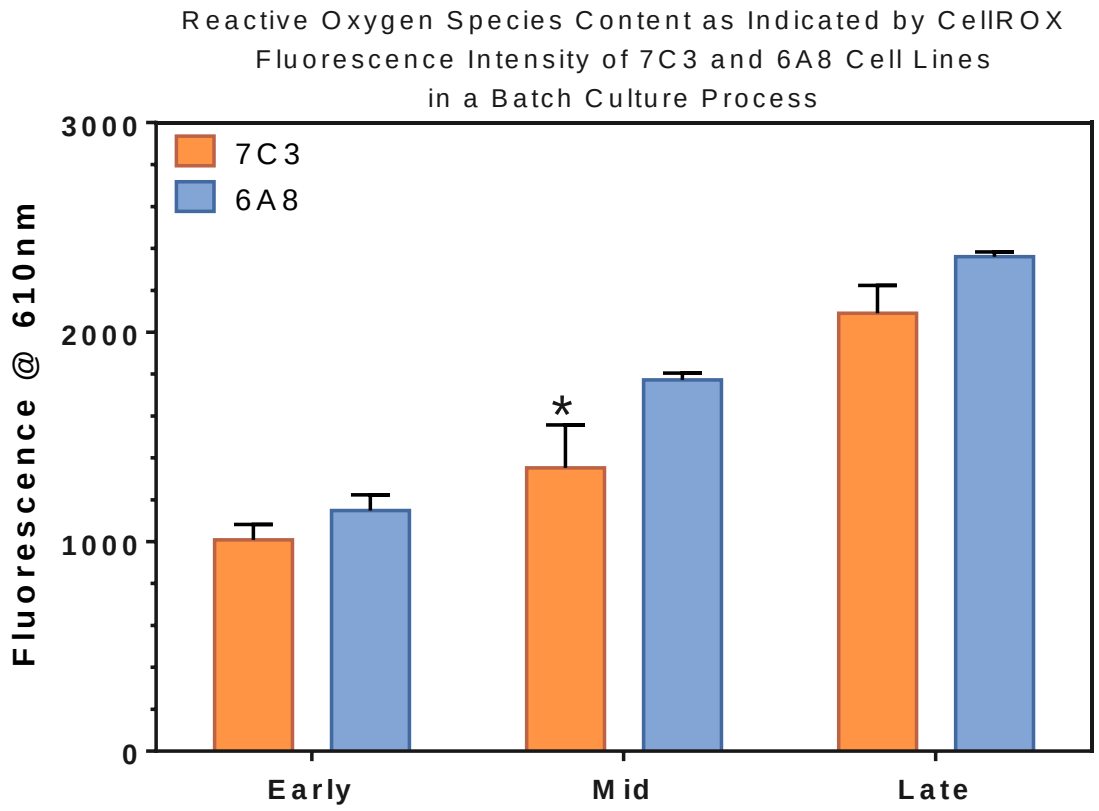

Figure 4.10. Intracellular ROS content of 7C3 and 6A8 producer cell lines in a batch process as measured at 72 h (Early), 120 h (Mid) and 168 h (Late) of culture indicated by Median Relative Fluorescence Units at 610 nm. Data points indicate the Mean average of samples taken from triplicate cell cultures and error bars indicate the Standard Error of the Mean. * indicates a significant difference between the cell lines ($p \leq 0.05$).

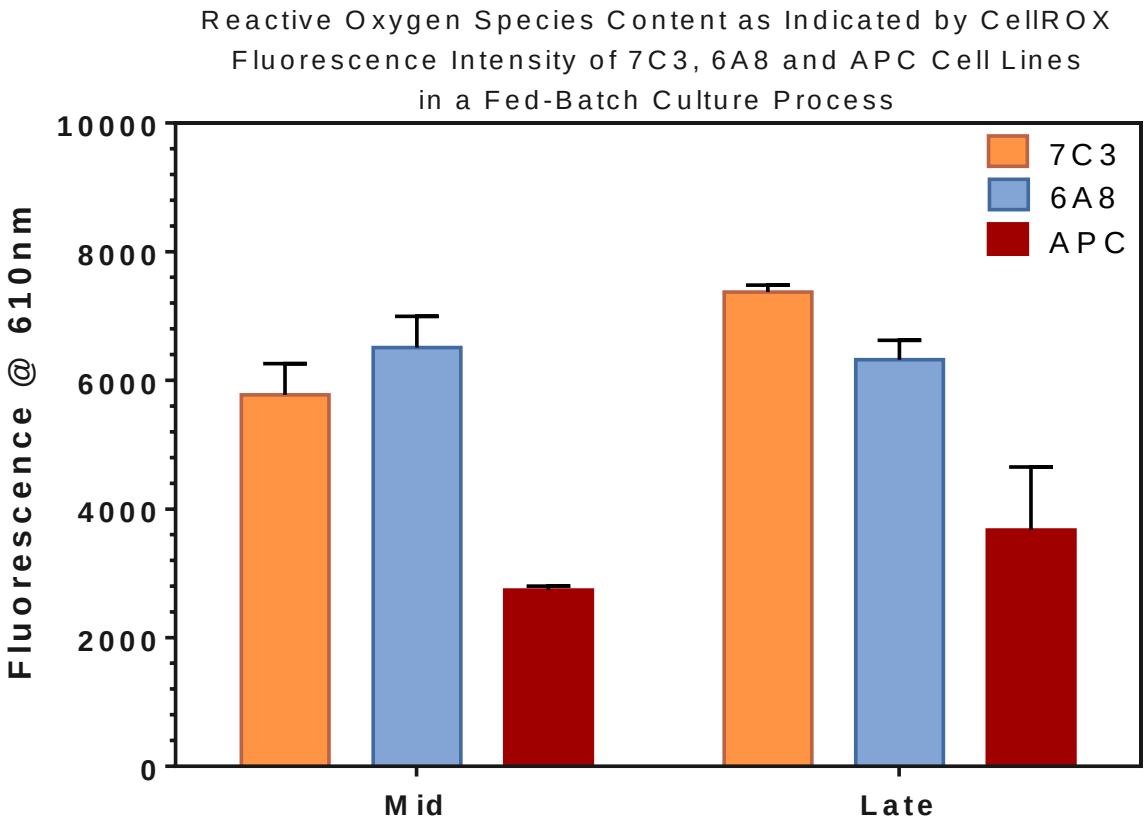

Figure 4.11. Intracellular ROS content of 7C3, 6A8 and APC producer cell lines in a batch process as measured at 144 h (Mid) and 264 h (Late) of culture indicated by Median Relative Fluorescence Units at 610 nm. Data points indicate the Mean average of samples taken from triplicate cell cultures and error bars indicate the Standard Error of the Mean.

Chapter Summary

Due to the extensive and often ill-documented experimentation using various 'strains' of Chinese Hamster Ovary cells, it is necessary to establish the relevant characteristics of a novel CHO cell line before embarking on any investigative study. Due to decades of genetic and phenotypic augmentation and drift, each individual cell line for each bioproduction process could be considered to be an individual 'qausispecies', and should be considered separate to all other cell lines, even those closely related to the line under investigation.

The cell lines used in this characterisation chapter were all familiarly connected by no more than three iterations of selection. Despite this, they often displayed differing characteristics in one or more of the monitored phenotypes. Prior to transfection and addition of the *DHFR* gene, the host cell line C2A that was the original progenitor of all producer cell lines showed much poorer growth characteristics, being unable to grow as quickly or last as long as the producer cell lines in a batch process. Once stable pools had been generated and clones selected in the first instance, 7C3 and 6A8 cell lines were able to grow for a further 48 hours and reach an IVCD nearly four times higher than that of the host. Metabolic analysis of the three cell lines indicated largely similar behaviour. Cell specific glucose consumption in 7C3 and 6A8 are almost identical, as are their specific lactate production profiles. Specific lactate production rates for the producer cell lines in a fed-batch process, however, were much lower than that of the host during batch production, and indeed lactate consumption began during fed-batch stationary phase; a phenomenon that did not occur in the host batch process. The producer cell line APC, whilst producing the same rMAb as the other two, represents a cell line created after an extra round of cloning and selection prior to transfection. It too was able to grow faster than the original host, but did not show any improved growth characteristics in comparison to the older cell lines.

In both batch and fed-batch cultures, 6A8 showed higher levels of productivity than 7C3. The higher rMAb titre when combined with the similar IVCD values shows that there is a greater cell specific productivity in 6A8 than 7C3, despite them both being derived from the same clonal pool and subjected to the same levels of development. APC was not analysed for productivity in a batch culture process, however in a fed-batch process it was vastly superior to the other two cell lines at producing the same easy to produce rMAb. When analysing the levels of ROS as determined by CellROX staining in all three cell lines, it is clear that APC has much lower levels of intracellular ROS than the other two. This increased resistance to oxidative stress could explain why there was such a greater level of productivity despite similar growth profiles. The ROS profiles of 7C3 and 6A8 are largely similar, and so the increased productivity seen in 6A8 again suggests it is a cell line that is more resistant to the detrimental effects of intracellular ROS accumulation.

All the cell lines represent CHO cell quasispecies that are very closely related, but that show key differences in growth, productivity and oxidative metabolism phenotypes. This, therefore, presents a situation where any investigation of their oxidative metabolism and its impact on their rates of productivity will also indicate whether adaptations to the oxidative state of cell lines could be generally applied, or would need to be much more cell line specific.

Chapter 5:

High-throughput Screening of Anti- and Pro-Oxidant Chemicals

Contents

List of Abbreviations

CHO	Chinese Hamster Ovary
H_2O_2	Hydrogen Peroxide
BSO	Buthionine Sulfoximine
ME	Menadione
CSI	Clone Select Imager
NADP/NADPH	Nicotinamide Adenine Dinucleotide Phosphate / Reduced NADP
IC_{50}/EC_{50}	50% Inhibition/Effective Concentration

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5.1 Introduction

As discussed in Chapter 2, Section 2.3.2 "*Antioxidant Chemicals*",pg19, the number of chemicals commonly used for the perturbation of the oxidative metabolism of cultured cells is large and incredibly varied. These chemicals differ substantially not only in their mechanism of action but in their efficacious dosage, which is also dependent on tissue type and culture method. There is very little evidence in the literature of studies utilising any anti-oxidants in CHO culture, and so it was necessary for the purposes of this study to identify appropriate concentrations for anti-oxidants to be trialled. Efficacious concentrations of anti-oxidant and pro-oxidant chemicals can range from the nanomolar to the millimolar, or even in some cases molar range. Therefore, a range of chemical concentrations need to be trialled for this mammalian system. Thus, it was necessary to identify an appropriate high-throughput method by which to screen useable concentrations of compounds rapidly and reliably. It is notable that is highly likely that the chemically defined media used to grow the cell lines in will already contain at least one, if not a combination of anti-oxidant chemicals in order to attempt to reduce the oxidative burden of the cells. However, manufacturers remain silent on whether or not these chemicals exist, and in what concentrations.

5.2 Experimentation

Host cell line C2A and producer cell lines 7C3 and 6A8 were revived and cultured during maintenance according to Chapter 3 Material and Methods Sections 3.2.4 "*Cell Revival*" pg36 and 3.2.2 "*Maintenance and Routine Sub-culture*", pg34. Anti-oxidant and Pro-oxidant chemicals used in screening and the concentration ranges screened are listed in 3.3.1 "*Chemicals Used for Screening*", pg39. Methodology for Presto-Blue® Cell Viability Assay and analysis is detailed in 3.3.2 "Presto-Blue® Analysis", pg32, and methodology for CellTOX™ Green Cytotoxicity Assay, Clone Select Imaging and analysis is detailed in 3.3.3 "*CellTOX™ Green Cytotoxicity Analysis*", pg40.

5.3 Presto-Blue® Cell Viability Assay

Initial screening using Presto-Blue® indicated promising results, with screens for pro-oxidants in C2A host cell line showing obvious dose dependent inhibition curves (Figure 5.1). In all three pro-oxidants screened, there did appear to be an increase in viability when concentrations became relatively high, a phenomenon that was most pronounced in C2A cells treated with Hydrogen Peroxide (H_2O_2). Despite the promising initial screens, it soon became clear that this assay was not suitable for screening of chemicals specifically intended to perturb the oxidative state of the cells.

Figure 5.2 shows images of 96 well plates containing producer cell lines treated with pro- and anti-oxidants during 3 days growth and then incubated for 30 minutes with Presto-Blue®. There

was a visible colour change in wells directly correlating with specific chemicals within the layout of the plate. Fluorescence intensity for these wells was 3 fold higher than the positive control, and reactions were dose independent.

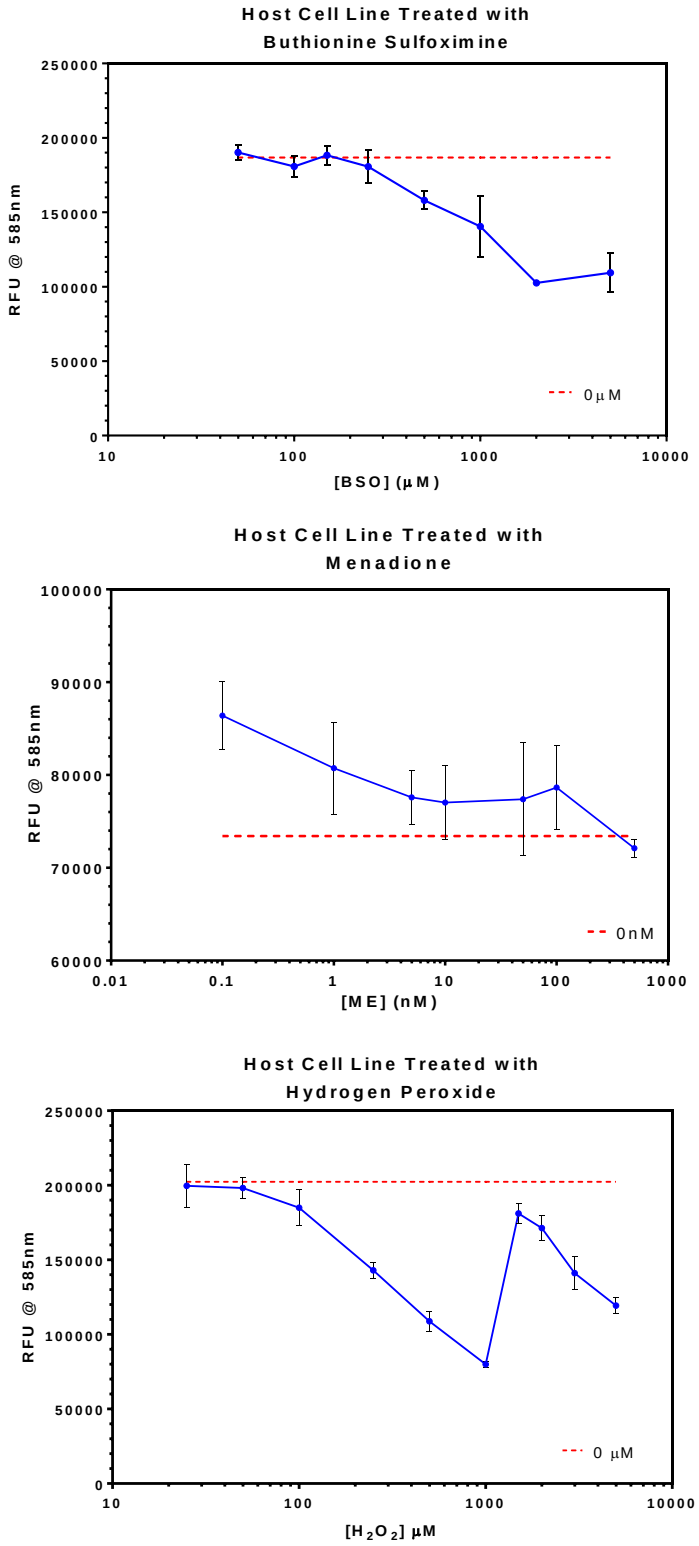

Figure 5.1. Inhibition curves for BSO, ME and H_2O_2 generated through use of Presto-Blue® Viability Assay. Data points indicate Mean Average of triplicate wells, with error bars indicating Standard Error of the Mean.

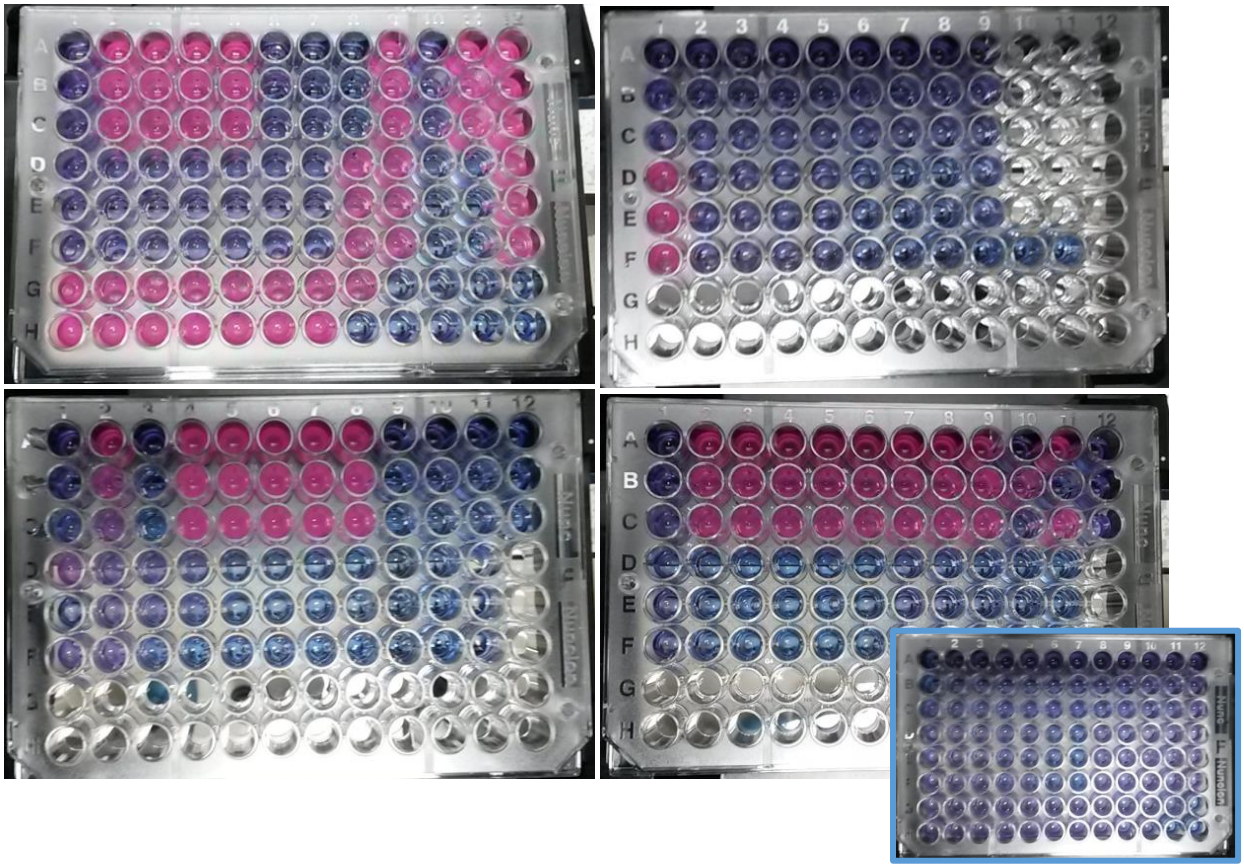

Figure 5.2. Screening plates incubated with Presto-Blue® showed aberrant colour changes on the visible range, with fluorescence readings 3 fold higher than positive control. Pink wells correlated directly with specific chemicals and colour changes were dose independent. **Inset:** a plate from an unrelated experiment screening chemicals not associated with oxidative metabolism.

Plates were imaged using the Clone Select Imager (CSI) in order to establish whether the fluorescence intensity was due to an unexpected bloom in growth, or indeed some sort of bacterial contamination. Figure 5.3 shows the confluence of such plates as determined by the CSI, compared with visual images of the plates. These comparisons showed a complete lack of growth in wells that correlated with the aberrant colour change, which is in direct contradiction of the methodology of the assay (higher fluorescence intensity = higher viability). The growth media and supplements and oxidative chemical carriers were tested to see if they had any contamination, or indeed if they reacted with Presto-Blue®, but all tests were negative. Laminar flow hoods and incubators used in the study were also contamination free, although this was unlikely to be the cause of the phenomenon as a contamination from an incubator would ordinarily produce a much more random infection pattern than was seen in these cases.

While Presto-Blue® was initially considered to be an uncomplicated assay to use for high throughput screening due to its ease of use and the extensive in house experience with it in the lab, the resazurin-based mechanism of the reagent was wholly inappropriate for use in the screening of chemicals that perturb the oxidative state of the cells. Resazurin utilises the cells' ability to reduce NADP to NADPH in order to fluoresce and give an indication of viability; any chemical that will alter the reducing power of the cells is likely to contradict and confuse any fluorescence from the reagent.

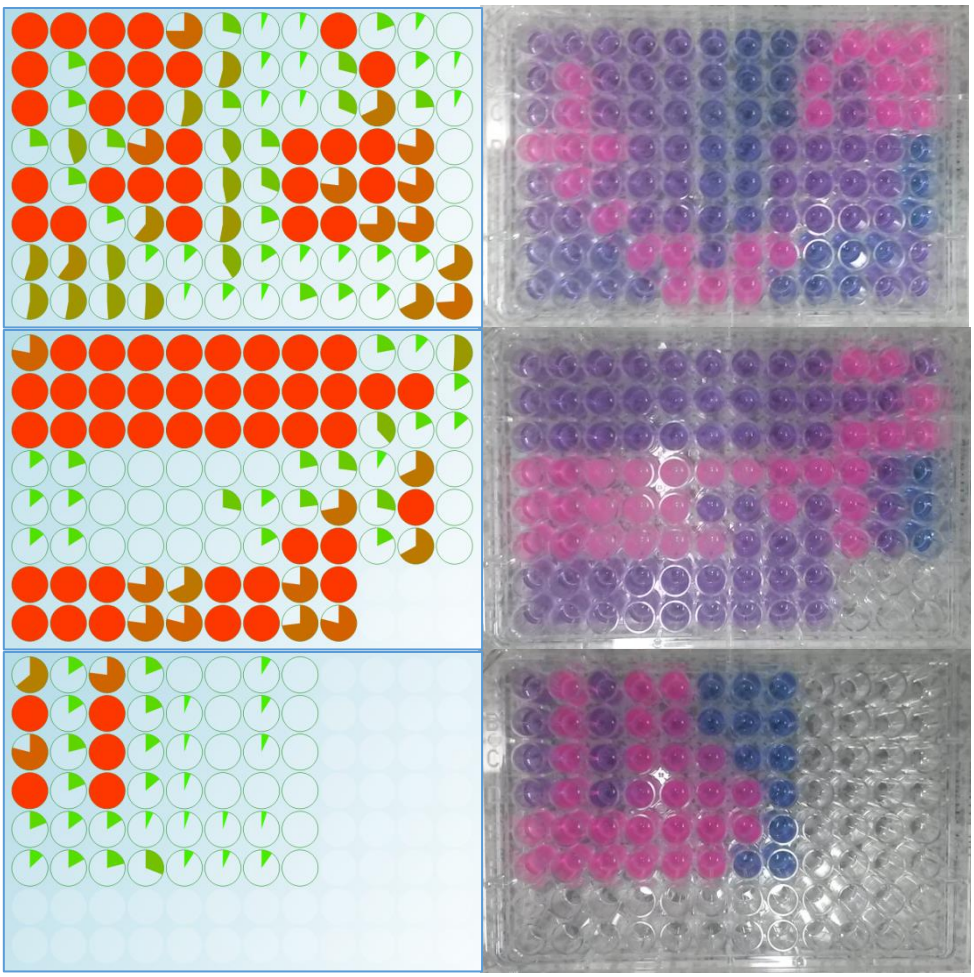

Figure 5.3. LEFT. Percentage confluence of screening plates as determined using the Clone Select Imager. **RIGHT.** Images of screening plates after incubation with PrestoBlue® Cell Viability Reagent.

5.4 Combining CellTOX™ and CSI as an Alternative Assay

It was therefore necessary to identify a plate-based assay that could be used to screen anti- and pro-oxidant chemicals in a high-throughput manner that did not rely on the reducing power of the cells to give an indicator of viability. A combinatorial approach was utilised in which a plate based imager

(the CSI) was coupled to a luminometric assay that does not rely on the reducing powers of the cell as its indicator. The CellTOX™ Green Cytotoxicity Assay from Promega uses an enzyme that luminesces when it comes into contact with DNA – an indicator that cells have lysed and are thus dead. It was therefore possible to monitor the growth of cells being treated with anti- or pro-oxidant chemicals while ensuring that the cell numbers observed correlated with live, healthy cells.

Figure 5.4 shows an example graph generated through the combination of these tools to illustrate how an appropriate concentration could be determined through this method, while a full catalogue of the compounds screened in producer cell lines 7C3 and 6A8 can be found in Appendix B. Cell Number data from the CSI can give an indication of growth, while CellTOX™ Green luminescence gives an indicator of the health of the cells. In pro-oxidant screening, it is possible to see when lower cell numbers correlates with cell death in a dose-dependent manner.

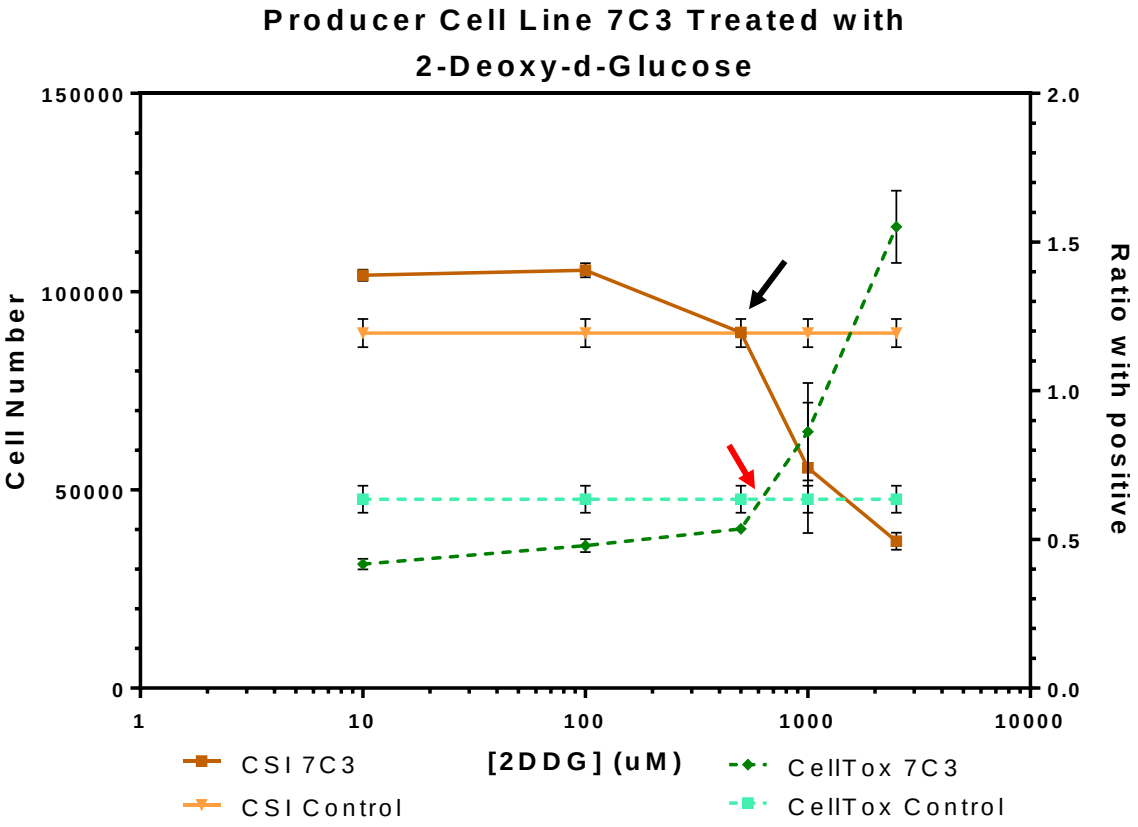

Figure 5.4. Example schematic of pro-oxidant chemical screened using the Clone Select Imager and CellTOX™ Green Cytotoxicity Assay. Producer cell line 7C3 was treated with increasing doses of 2-Deoxy-D-Glucose (2DDG). At 750 µM the cell number can be seen to drop below the cell number of untreated cells (Black Arrow). At 800 µM the luminescence of treated cells exceeded that of the untreated cells (Red Arrow). There is little difference in Cell Number between 10 µM and 100 µM, however there is an increase in luminescence, suggesting a decrease in viability. The working concentration thus lies between 100 µM and 800 µM. Data points indicate the Mean Average of 6 wells over three plates, with error bars indicating Standard Error of the Mean.

In anti-oxidant screening, it is possible to see any dose-dependent changes in growth while ensuring that such concentrations of anti-oxidant are not also cytotoxic.

5.4.1 Anti-Oxidant Compounds in Producer Cell Lines

Table 5.1 shows the concentrations of anti-oxidants identified as the working concentration based on the combined CSI and CellTox™ data.

Table 5.1. Anti-oxidant compounds and the working concentrations used based on combined data from CSI and CellTox™ analysis.

Anti-Oxidant Compound	Working Concentration
Ascorbic Acid	100 µM
α-Lipoic Acid	25 µM
Butylated Hydroxyanisole	1 µM
Glutathione	50 µM
L-Carnosine	10 mM
MitoQ	10 nM
MitoTEMPOL	5 nM
N-Acetylcysteine	1 mM
Procysteine	1 mM
Sodium Selenite	100 nM

5.4.2 Pro-Oxidant Compounds in Producer Cell Lines

Table 5.2 shows the concentrations of pro-oxidants identified as the working concentration based on the combined CSI and CellTox™ data.

Table 5.1. Pro-oxidant compounds and the working concentrations used based on combined data from CSI and CellTox™ analysis.

Pro-Oxidant Compound	Working Concentration
Buthionine Sulfoximine	500 µM
2-Deoxy-d-Glucose	750 µM
Hydrogen Peroxide	250 µM
Menadione	5 nM

Chapter Summary

High-throughput screening is a powerful tool for rapidly identifying appropriate concentrations of test compounds, however care must be taken when selecting the assay methodology in order to avoid confusing the data. A commonly used biochemical method of measuring cell viability is the resazurin based Presto-Blue® assay, however this is inappropriate for a study involving compounds that perturb the oxidative state due to its reliance on the reductive power of the cells as an indicator of viability. This study instead utilised the imaging and in-built cell number algorithms of the Clone Select Imager to monitor the growth of the cells during screening, in combination with the Promega CellTox™ Green Cytotoxicity Assay for monitoring of viability.

Appropriate working concentrations for anti- and pro-oxidant compounds were thus identified through screening for cell number and viability (indicators of cell growth) after 3 days in static plate based culture for use in more mid-throughput screening of compounds when used in shaking flask cultures in either batch or fed-batch processes.

Chapter 6a:

Anti- and Pro-oxidant Supplementation in Batch Culture Processes

Contents

List of Abbreviations

PO	Pro-Oxidant	q_P	Cell Specific Productivity
H_2O_2	Hydrogen Peroxide	AO	Anti-Oxidant
BSO	Buthionine Sulfoximine	AA	Ascorbic Acid
ME	Menadione	AL	α-Lipoic Acid
2DDG	2-Deoxy-d-Glucose	SS	Sodium Selenite
ROS	Reactive Oxygen Species	MQ	MitoQ
$O_2^{\bullet}$	Superoxide Anion	MT	MitoTEMPOL
SOD	Superoxide Dismutases	NAC	N-Acetylcysteine
GCL	Glutamate Cysteine Ligase	GSH	Glutathione
IVCD	Integral Viable Cell Density	BHA	Butylated Hydroxyanisole
rMAb	Recombinant Monoclonal Antibody	OTZ	Procysteine
UPR	Unfolded Protein Response	LCA	L-Carnosine

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6a.1 Introduction

To gain a greater understanding of the oxidative defence mechanisms that underpin different producer cell lines, it was necessary to introduce pro-oxidant chemicals. For example, if the cells' biomanufacturing phenotypes were less impacted when being challenged with Hydrogen Peroxide (itself a Reactive Oxygen Species (ROS)) but not Buthionine Sulfoximine (an inhibitor of the non-redundant Glutathione production pathway), it would suggest that the cells have adequate scavenging mechanisms (and so can cope with an instant oxidative challenge), but are unable to recover from or adapt to an assault on the production of glutathione.

Further understanding of the oxidative nature of producer cell lines is possible through supplementation with anti-oxidant chemicals. Evidence in the literature suggests that anti-oxidant compounds have varying degrees of efficacy depending on the tissue or cell type, suggesting that different cell types benefit from different oxidative support strategies. By introducing compounds to the growth media with a range of anti-oxidant mechanisms, it will be possible to have a greater understanding of the oxidative metabolism of the producer cell lines, and thus have a more targeted approach to developing feeding strategies using anti-oxidants to enhance cellular biomanufacturing performance through boosting their oxidative capacity. Since productivity is highest during the stationary phase of growth, and also one of the major sources of cellular ROS, it may be that introducing some anti-oxidant compounds at the end of exponential phase would have more of an impact on overall cellular productivity.

6a.2 Experimentation

Producer cell lines 7C3 and 6A8 were cultured in a batch culture process as detailed in Chapter 3: Materials and Methods section 3.2.2 *"Maintenance and Routine Sub-culture"*, pg34. For determination of growth and productivity, cells were grown in 50mL vented Bioreactor Cultiflasks (Sartorius AG, Göttingen, Germany) at 200 rpm with 15 mL culture volume, at 37 °C, 5 % (v/v) CO_2. For determination of cellular Reactive Oxygen Species levels, cells were grown in 125 mL Erlenmeyer Flasks with Vent Cap (Corning®, Tewksbury, US) at 140 rpm with 30 mL culture volume, at 37 °C, 5 % (v/v) CO_2, 80 % humidity.

Concentrations of Anti-oxidant chemicals used are detailed in Table 3.2, Section 3.4 *"Batch and Fed-Batch Screening of Anti- and Pro-Oxidants",* pg41. Viable cell density and culture % viability were determined using a Vi-CELL® Cell Viability Analyser (Beckman Coulter, High Wycombe, UK), according to 3.2.2 *"Maintenance and Routine Sub-culture"*, pg34. Integral Viable Cell Density (IVCD) was calculated using Equation 3.1, pg35. Product titre was measured according to 3.2.5

"Productivity", pg36, and cell specific productivity (q_P) was calculated according to Equation 3.5, p37. Reactive Oxygen Species content was determined according to 3.4.3 *"Reactive Oxygen Species Quantification"*, pg43. All graphs were generated using GraphPad Prism and statistical significance was calculated using multiple paired t-tests.

6a.3 Mechanistic Exploration of Cellular Oxidative Defences

Whilst all mammalian cells have an extensive network of anti-oxidant defence systems, evolution and genetic drift during routine sub-culture combined with differing environmental pressures may result in the relative strength of these defences differing between cell types, and even cell lines. To probe the cellular anti-oxidant defence systems of the two producer cell line, 7C3 and 6A8, chemicals designed to increase the oxidative burden were introduced to culture medium in a batch culture process. In separate experiments, these pro-oxidant (PO) chemicals were introduced on the day of inoculation (Day0) and at late exponential phase (Day4), to establish whether the nature of the oxidative defences was different at different periods in cell culture. The four chemicals used were Hydrogen Peroxide (H_2O_2), Buthionine Sulfoximine (BSO), Menadione (ME) and 2-Deoxy-d-Glucose (2DDG).

Hydrogen Peroxide is a Reactive Oxygen Species (ROS) molecule and so was introduced to culture with a view to directly increasing the overall oxidative burden on the cells. Similarly, when metabolised, the well-used quinone Menadione is known to be reduced ultimately to a superoxide anion ($O_2^{\bullet}$) that increases the cellular oxidative burden. H_2O_2 and $O_2^{\bullet}$ are reduced through differing mechanisms within the cell; H_2O_2 is converted to water and oxygen molecules by the enzymes caspase and glutathione peroxidase, whereas $O_2^{\bullet}$ is reduced to H_2O_2 by superoxide dismutases (SODs), to then be reduced as previously described. An inhibitor of Glutamate Cysteine Ligase (GCL, formerly γ-Glutamylcysteine Synthetase), the enzyme responsible for the rate-limiting step in glutathione synthesis, BSO directly impacts the cells' ability to replenish its glutathione pool. 2-Deoxy-D-Glucose is a competitive inhibitor of glucose uptake, and is associated with increasing incidence of oxidative stress through reducing production of both pyruvate (from glycolysis) and NADPH (from the pentose pathway).

6a.3.1 Effect of Pro-oxidants on Growth

Figure 6a.1 shows the IVCD values for producer cell lines 7C3 and 6A8 treated with H_2O_2, BSO, ME and 2DDG separated into "Early" (72h), "Mid" (120h) and "Late" (196h) stages of growth. As discussed earlier, there are metabolic shifts associated with the transition from exponential growth to stationary growth such as the transition from Warburg metabolism to Oxidative Phosphorylation,

and again onto late stationary growth and death, and so the decision was made to focus on these three key points in the culture period. When introduced to the cell culture medium on the day of inoculation, H_2O_2, BSO and 2DDG all had a detrimental effect on the growth of both 7C3 and 6A8 cell lines, with final IVCD values being less than half of that of the untreated cell lines in all cases(Figure 6a.1A and B). There is a significant ($p<0.001$) reduction in late stage growth in both cell lines when treated with these three chemicals. 2DDG had no impact on mid-stage growth of the 7C3 cell line, but there is a significant reduction in mid-stage growth when treated with H_2O_2 and BSO ($p<0.001$), and for all three in the 6A8 cell line ($p<0.001$). Interestingly, despite the drastic reduction in $IVCD_{MAX}$, exponential growth was not impacted negatively or positively by any of the chemicals in either cell line. Menadione appeared to have little to no impact on the growth of these cell lines in either case, with only a slight reduction in $IVCD_{MAX}$ for the 6A8 cell line ($p<0.05$) from 685 cell•h/mL to 604 cell•h/mL.

In contrast to the large negative impact on growth when introduced at the beginning of culture, the introduction of PO at the end of exponential growth had no effect. For the 7C3 producer cell line (Fig. 6a.1.C), it was observed that H_2O_2, BSO and ME had no impact on cell growth, whilst 2DDG had a positive impact on $IVCD_{MAX}$, with an increase from 691 cell•h/mL to 796 cell•h/mL ($p<0.01$). Figure 6a.1.D indicates that there is also no impact on mid-stage growth when treated with any of the PO, and 2DDG and H_2O_2 had no impact on overall growth. Menadione again had a slight negative impact on late stage growth, with a drop in $IVCD_{MAX}$ to 651 cell•h/mL ($p<0.1$). BSO also had a negative impact on $IVCD_{MAX}$, dropping to 613 cell•h/mL ($p<0.001$).

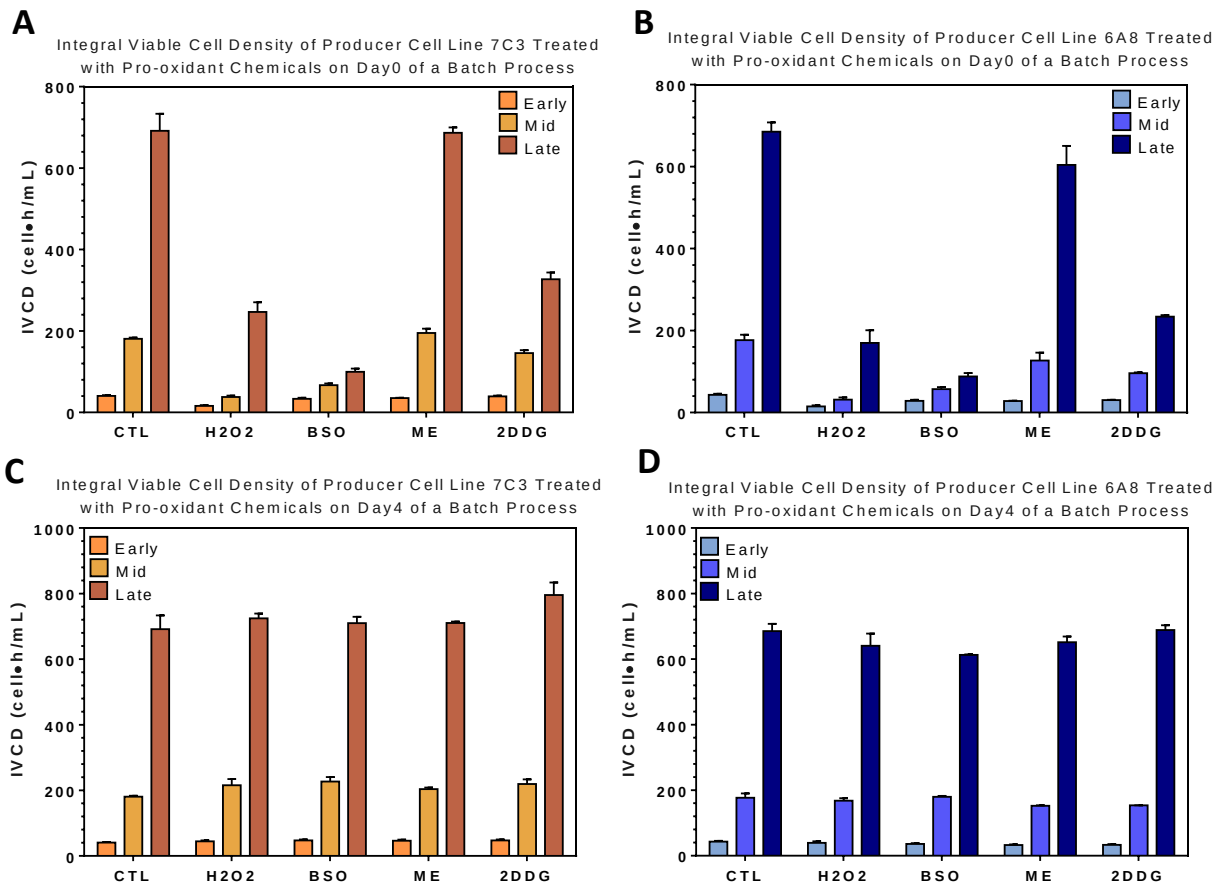

Figure 6a.1. Integral Viable Cell Density of 7C3 and 6A8 producer cell lines at 72h ("Early"), 120h ("Mid") and 192h ("Late") of a batch culture process when treated with pro-oxidant chemicals Hydrogen peroxide (H2O2), Buthionine Sulfoximine (BSO), Menadione (ME) and 2-Deoxy-d-Glucose (2DDG) on (A,B) Day0 of culture and (C,D) Day4 of culture. Corresponding data for untreated cells is shown in "CTL" bars. Data bars represent Mean average of samples taken from triplicate cultures and error bars indicate Standard Error of the Mean.

6a.3.2 Effect of Pro-oxidants of Productivity

Both 7C3 and 6A8 are producing the same recombinant monoclonal antibody (rMAb) that represents an easy-to-express protein similar to many produced in the biologics industry. Protein folding and secretion are known to increase production of ROS (Kim *et al*, 2008), and it is also known that oxidative stress is linked to the Unfolded Protein Response (UPR) and UPR-induced apoptosis (Santos *et al*, 2009; Du *et al*, 2013). Pro-oxidants were used to probe the cells' production response to an increase in oxidative stress, and to establish when in cell culture changes to oxidative pressures are more likely to affect productivity. Figure 6.2 shows the relative final day rMAb titre of the producer cell lines treated with PO as a ratio to that of untreated cells. As with IVCD, the greatest impact on productivity was seen when PO were added in the inoculation medium, rather than at the end of the exponential phase of growth. Hydrogen peroxide introduced on Day0 decreased final titre of 7C3 by 52 % and of 6A8 by 64 %, and when treated with BSO final day titre was reduced by 21 % and 42 %, respectively (Fig.6a.2.A).

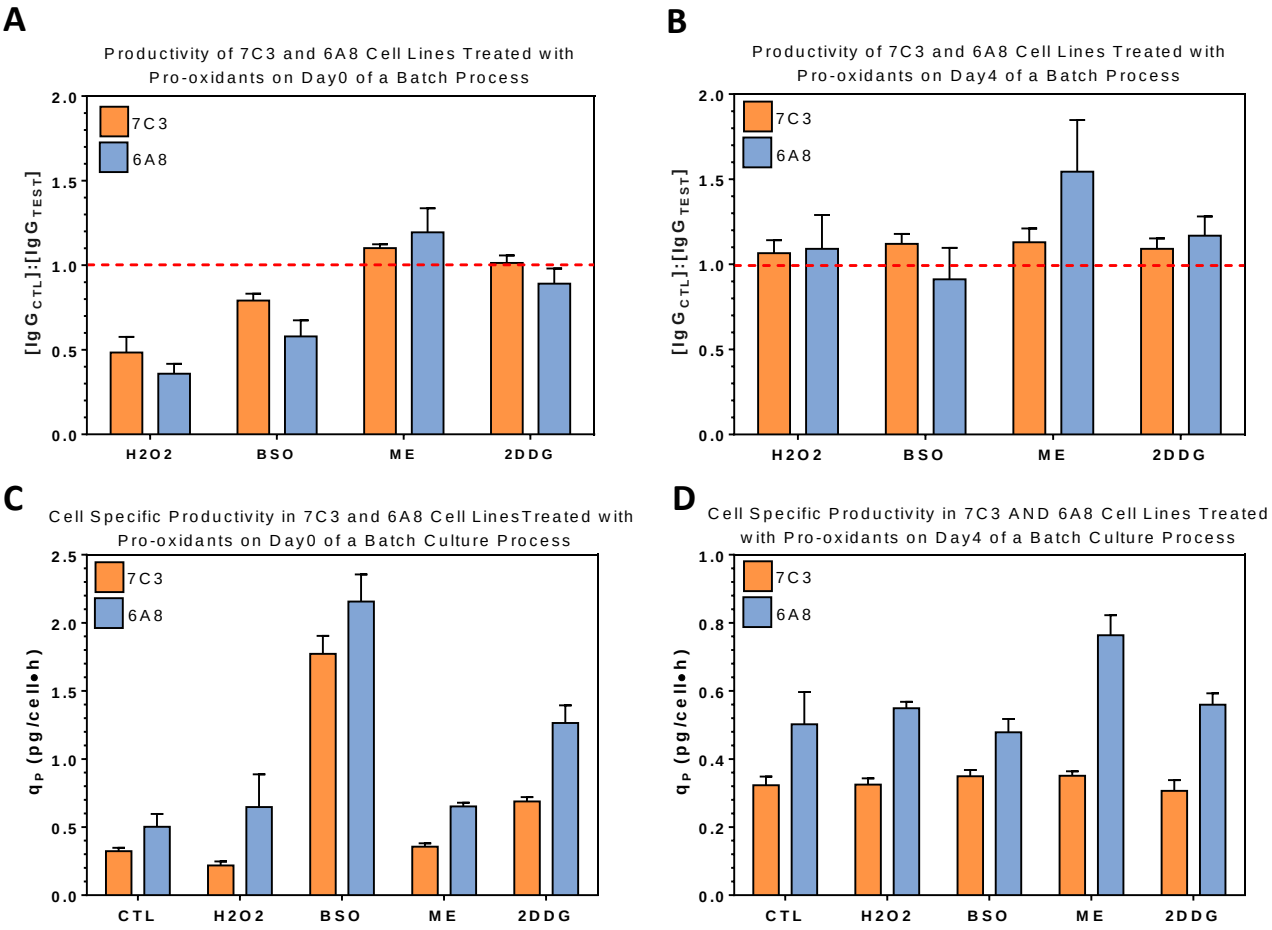

Figure 6a.2. Productivity of 7C3 and 6A8 producer cell lines in a batch culture process when treated with pro-oxidant chemicals (PO) Hydrogen peroxide (H2O2), Buthionine Sulfoximine (BSO), Menadione (ME) and 2-Deoxy-d-Glucose (2DDG). Data bars represent Mean average of samples taken from triplicate cultures and error bars indicate Standard Error of the Mean. (A) Final day titre when treated with PO on Day0 of culture. (B) Final day titre when treated with PO on Day4 of culture. (C) Cell specific productivity at 192h when treated with PO on Day0 of culture. (D) Cell specific productivity at 192h when treated with PO on Day4 of culture. (C,D) Corresponding data for untreated cells is shown in "CTL" bars.

Conversely, Menadione and 2DDG had no impact on the productivity of 7C3; 6A8 saw a slight increase ($p<0.1$) in productivity when treated with Menadione but no change when treated with 2DDG. In contrast to these data, when treated with the same concentration of PO chemicals on the fourth day of culture (i.e. at the end of exponential growth), there were no significant changes to final product titre in either cell line for any of the chemicals used, save for a slight increase in titre for the 6A8 cultures treated with ME (an increase of an extra 54 %, $p<0.05$) (Fig. 6a.2.B).

When analysing cell specific productivity (q_P), the most stark observation is the large increase in q_P when both cell lines are treated with BSO on Day0 of culture (an increase from 0.323 pg/cell•h to 1.77 pg/cell•h and from 0.502 pg/cell•h to 2.16 pg/cell•h in 7C3 and 6A8, respectively) (Fig.6a.2.C). This observation is likely due to the fact that growth was still substantially reduced by the end of culture, and while titre was negatively impacted it was proportionally less so, indicating

an overall increase in q_P in comparison to the untreated culture. Cultures treated on Day0 with 2DDG were similarly affected, and an increase of q_P to 0.689 pg/cell•h and 1.26 pg/cell•h in 7C3 and 6A8, respectively was observed. Cell specific productivity in cultures treated with H_2O_2 on Day0 was not significantly impacted, and only 6A8 cultures treated with ME were different to that of the untreated cultures (an increase in q_P to 0.652 pg/cell•h, $p<0.1$). In comparison, mirroring the growth and titre data observed, there is no impact on q_P when treating the cell lines with PO on Day4 of culture, except for a slight increase in the case of ME treated 6A8 (from 0.502 pg/cell•h to 0.764 pg/cell•h, $p<0.05$)(Fig.6.2.D).

6a.4 Media Supplementation with Anti-oxidants in a Batch Culture Process

Further to the mechanistic probing of cell lines using chemicals designed to increase the oxidative burden, it is also possible to introduce anti-oxidant (AO) chemicals in order to gauge which areas of the cellular defences benefit most from environmental support. This knowledge can then feed into the development of feeding strategies aimed at improving cell line performance. Finally, producer cell lines 7C3 and 6A8 were grown in a batch process and supplemented with various anti-oxidant chemicals on either Day0 (inoculation) or Day4 (end of exponential) at concentrations determined in Chapter 5 ("*Anti-Oxidant Compounds in Producer Cell Lines*", pg82). Cells were then assessed for their intracellular levels of Reactive Oxygen Species (ROS), their growth and their productivity. The anti-oxidants used, their abbreviations and their concentrations are summarised again in Table 6a.1.

Table 6a.1. Anti-oxidant chemicals used in Batch Culture experimentation, with appropriate abbreviations and concentrations used.

Anti-Oxidant Compound	Abbreviation	Concentration Used
Ascorbic Acid	AA	100 µM
α-Lipoic Acid	AL	25 µM
Butylated Hydroxyanisole	BHA	1 µM
Glutathione	GSH	50 µM
L-Carnosine	LCA	10 mM
MitoQ	MQ	5 nM
MitoTEMPOL	MT	10 nM
N-Acetylcysteine	NAC	1 mM
Procysteine	OTZ	1 mM
Sodium Selenite	SS	100 nM

6a.4.1 Cellular ROS Levels after Anti-oxidant Supplementation

Intracellular ROS levels as determined by CellROX staining and flow cytometry analysis are shown in Figure 6a.3 as a ratio with untreated cell samples. Cell samples were obtained at 72h ("Early"), 120h ("Mid") and 168h ("Late") of a batch culture process.

6a.4.1.2 Supplementation at Inoculation

In exponential growth, it was observed that Day0 treatment with Ascorbic Acid (AA), α-Lipoic Acid (AL) and Sodium Selenite (a water soluble form of selenium, SS) led to a reduction in intracellular ROS levels in 7C3 cultures (AA and SS $p<0.05$, AL $p<0.01$)(Fig.6.3.A). Additionally, there was a general downward trend in ROS levels of 7C3 cells treated on Day0 with MitoQ (MQ), MitoTEMPOL (MT) and N-Acetylcysteine (NAC), although these data did not show a statistically significant change compared to untreated cells. Similarly to 7C3, Day0 treatment with AA resulted in a reduction of intracellular ROS levels of 6A8 cells in exponential phase ($p<0.05$), as well as downward trends seen when treated with MQ, NAC and SS (Fig.6a.3.B). Interestingly, ROS levels in both cell lines were increased in exponential phase when treated with Glutathione (GSH) on Day0, with 6A8 cells seeing a significant increase in comparison to untreated cells ($p<0.01$).As the cells progressed into mid-stationary phase, 7C3 cultures treated with AL continued to see a reduction in ROS levels ($p<0.05$) although to a lesser degree than earlier in culture, seeing a reduction of only 5 % in comparison with the 23 % reduction seen at 72 h. 7C3 cells treated with MT also saw a slight reduction in ROS levels during mid-stationary phase ($p<0.1$) with an observed drop of 28 %, whilst cells treated with NAC showed an increase of 33 % ($p<0.01$), contrary to the reduction seen earlier in culture. Treatment with AA and GSH on Day0 of culture continued to result in a reduction in ROS levels of 6A8 cultures as they progressed to mid-stationary phase (a 45 % and 39 % reduction, respectively), along with a 33 % reduction in cultures treated with AL ($p<0.1$) and a 31 % reduction in those treated with MT ($p<0.05$). Both cell lines continued to see a reduction in ROS levels in cultures treated with SS.

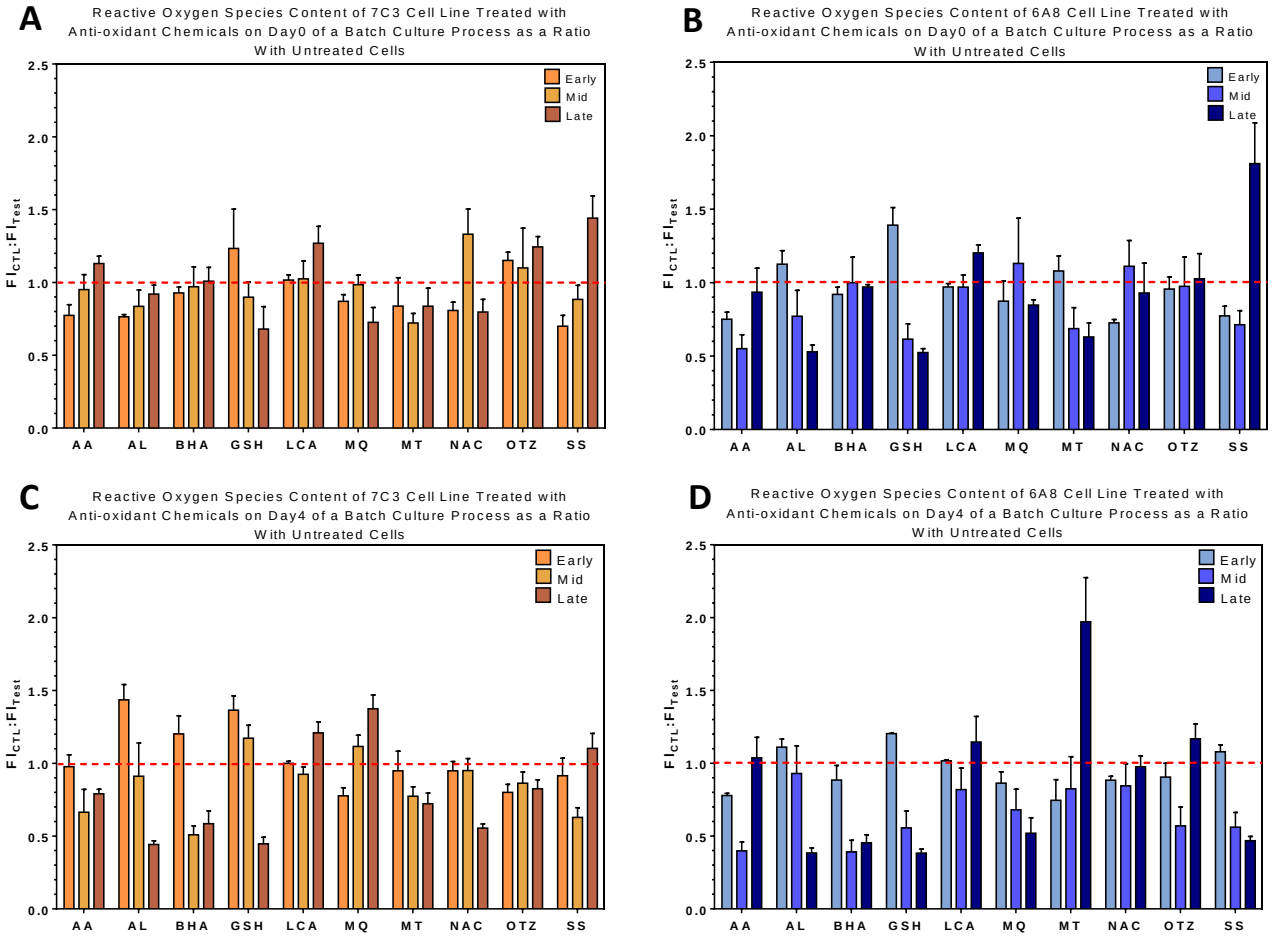

Figure 6.3. Reactive Oxygen Species levels of 7C3 and 6A8 producer cell lines in a batch culture process when treated with anti-oxidant chemicals Ascorbic Acid (AA), α-Lipoic Acid (AL), Butylated Hydroxyanisole (BHA), Glutathione (GSH), L-Carnosine (LCA), MitoQ (MQ), MitoTEMPOL (MT), N-Acetylcysteine (NAC), Procysteine (OTZ) and Selenium (SS). Relative ROS levels are shown as a ratio of CellROX stained cells' Median fluorescence at 610nm with that of untreated cells, and are shown at 72h ("Early"), 120h ("Mid"), and 168h ("Late") of culture. Data bars represent Mean average of samples taken from triplicate cultures and error bars indicate Standard Error of the Mean. (A) ROS levels of 7C3 when treated with AO on Day0 of culture. (B) ROS levels of 6A8 when treated with AO on Day0 of culture. (C) ROS levels of 7C3 when treated with AO on Day4 of culture. (D) ROS levels of 6A8 when treated with AO on Day4 of culture.

The reduction in ROS levels in 7C3 cells treated with AL did not continue into late stationary phase, nor did cells treated on Day0 with MT. Cells treated with MQ, however, did display a late-culture-stage reduction of 27 % in cellular ROS levels ($p<0.01$), and those treated with GSH had a late-stage-culture reduction of ROS of 32 % ($p<0.1$, respectively). 7C3 cells treated on Day0 with L-Carnosine (LCA) and SS saw an increase in ROS at 168 h of 27 % ($p<0.05$) and 44 % ($p<0.01$), respectively. 6A8 cell lines treated on Day0 saw a late-stage ROS reduction when treated with AL,

GSH and MT, with reductions of 47 %, 48 % and 37 %, respectively ($p<0.01$). A pattern not observed earlier in culture, 6A8 cells treated on Day0 with LCA and SS displayed a marked increase in ROS levels at 168 h, with an increase of 20 % and 82 %, respectively ($p<0.01$).

6a.4.1.2 Supplementation at Late Exponential Growth

Cellular ROS content changes when supplemented with AO chemicals at the end of exponential growth, rather than at inoculation, indicate that there are definite differences between the impact of introducing chemicals at different times of culture, thus suggesting that the nature of oxidative burden changes as the cells progress through culture.

For cell line 7C3, it was observed that at 120h there was a significant decrease in cellular ROS levels in cultures treated with AA, Butylated Hydroxyanisole (BHA), MT and SS, with decreases observed of 34 %, 49 %, 23 % and 37 %, respectively ($p<0.05$), whilst there was a slight increase of 17 % in those cultures treated with GSH ($p<0.1$). There was no reduction seen in cultures treated with AL, nor in those treated with NAC, indicating a difference from cell cultures treated with these chemicals on Day0. As cells progressed into late-stationary phase, the reduction in ROS continued in cultures treated with AA, BHA and MT, with observed decreases in ROS of 21 % ($p<0.1$), 41 % and 28 % ($p<0.05$), respectively. Additionally, there were now reductions of 56 %, 55 %, 45 % and 18 % in ROS levels observed in those cultures treated with AL, GSH, NAC and Procysteine (OTZ), respectively ($p<0.05$). Similarly to those cultures treated with AO on DAY0, there was an increase in ROS levels in those cultures treated with LCA, with a similar increase of 21 % ($p<0.01$). There was also an increase in ROS levels in cultures treated with MQ, as these cultures showed a 37 % increase ($p<0.01$) at late-stationary phase.

At mid-stationary phase, intracellular levels of ROS in 6A8 cultures were decreased in those treated with AA, BHA, GSH, MQ, OTZ and SS. While AA and GSH resulted in a mid-stationary drop in ROS cultures when included on Day0, the reduction seen when using these chemicals at Day4 was more pronounced, with these later treated cultures seeing a 60 % and 45 % drop, respectively ($p<0.01$). Most of the other chemicals eliciting a negative influence on ROS levels had a similarly pronounced effect, with BHA, MQ, OTZ and SS seeing reductions of 60 %, 32 %, 43 % and 44 %, respectively ($p<0.01$ for BHA, OTZ, SS, $p<0.05$ for MQ). There were no AO chemicals added on Day4 of culture that elicited an increase in cellular ROS levels at mid-stationary phase. As the cells progressed into late-stationary phase, cultures treated with BHA, GSH, MQ and SS on Day4 of culture continued to display a decrease in ROS levels, and with reductions of 62 %, 62 %, 48 % and 53 % respectively ($p<0.01$), these cultures actually saw a continued decline in ROS levels through culture despite the usual pattern of ROS accumulation in older cultures. Cultures treated with AL on Day4 also saw a continual decline in ROS levels throughout culture, with measurements at late-stationary

phase showing a 62 % reduction ($p<0.01$) of ROS compared to untreated cultures. Conversely, 6A8 cultures treated with MT on Day4 saw a large increase in ROS levels on Day4, with a substantial 97 % increase in comparison with untreated cultures ($p<0.01$).

6a.4.2 Impact of Anti-Oxidant Treatment on Cellular Growth

While anti-oxidant treatment manipulated Reactive Oxygen Species levels throughout culture, this change in ROS levels is merely academically interesting without it translating into beneficial bioprocess phenotypes such as growth or productivity. One characteristic of particular interest is cellular growth throughout culture, and so cell cultures treated with AO on Day0 and on Day4 of batch cultures were analysed for their Integral Cell Density at 72 h ("Early"), 120 h ("Mid") and 168 h ("Late") of growth. Figure 6a.4 shows IVCD of producer cell lines 7C3 and 6A8 at these time points when treated on Day0 (Figa.6.4.A,B) and Day4 (Figa.6.4.C,D) of culture.

When treating producer cell cultures at the point of inoculum with AO chemicals, there were no immediate impacts on the growth of the cells, with IVCD values for both treated 7C3 and treated 6A8 cultures showing no marked difference to those of the untreated control cultures. There was similarly very little impact on mid-stationary phase growth. In both 7C3 and 6A8 cultures treated with GSH there was a significant increase in IVCD, with values for 7C3 cultures increased to 271 cell•h/mL compared to the control IVCD of 173 cell•h/mL ($p<0.01$); GSH treated 6A8 cultures had an IVCD 211 cell•h/mL compared to the control cultures' IVCD of 148 cell•h/mL ($p<0.01$).

The only other AO chemical to have an impact on mid-stationary growth of the producer cell lines when introduced on Day0 of culture was BHA, with an observed increase in 7C3 IVCD to 254 cell•h/mL. As the cultures progressed to late-stationary phase, many more of the chemicals had a significant impact on IVCD. In 7C3 cultures treated on Day0, there was an increase in IVCD in those cultures treated with AL, BHA, GSH, LCA, MQ, MT and OTZ, with these cultures showing IVCD values of 804 cell•h/mL, 774 cell•h/mL, 914 cell•h/mL, 739 cell•h/mL, 828 cell•h/mL, 817 cell•h/mL, and 749 cell•h/mL, respectively ($p<0.01$ for all except LCA ($p<0.1$) and OTZ ($p<0.05$)), compared to the late-stationary phase IVCD of the original 7C3 cultures of 691 cell•h/mL. 6A8 cultures treated with GSH, LCA and MT on Day0 also saw increased IVCD values at late-stationary phase of growth, with observed values for these cultures of 832 cell•h/mL, 741 cell•h/mL, and 759 cell•h/mL, respectively ($p<0.05$), compared to the untreated IVCD of 685 cell•h/mL. Treatment with NAC on Day0 of culture had a negative impact on the late-stationary growth of both cell lines, with observed IVCD for those treated cultures of 627 cell•h/mL ($p<0.05$) and 593 cell•h/mL ($p<0.01$) for 7C3 and 6A8 cultures, respectively. 6A8 cultures treated on Day0 AA and MQ also displayed a reduced IVCD later in culture,

with observed values of 604 cell•h/mL for cultures treated with AA and of 596 cell•h/mL for MQ ($p<0.01$).

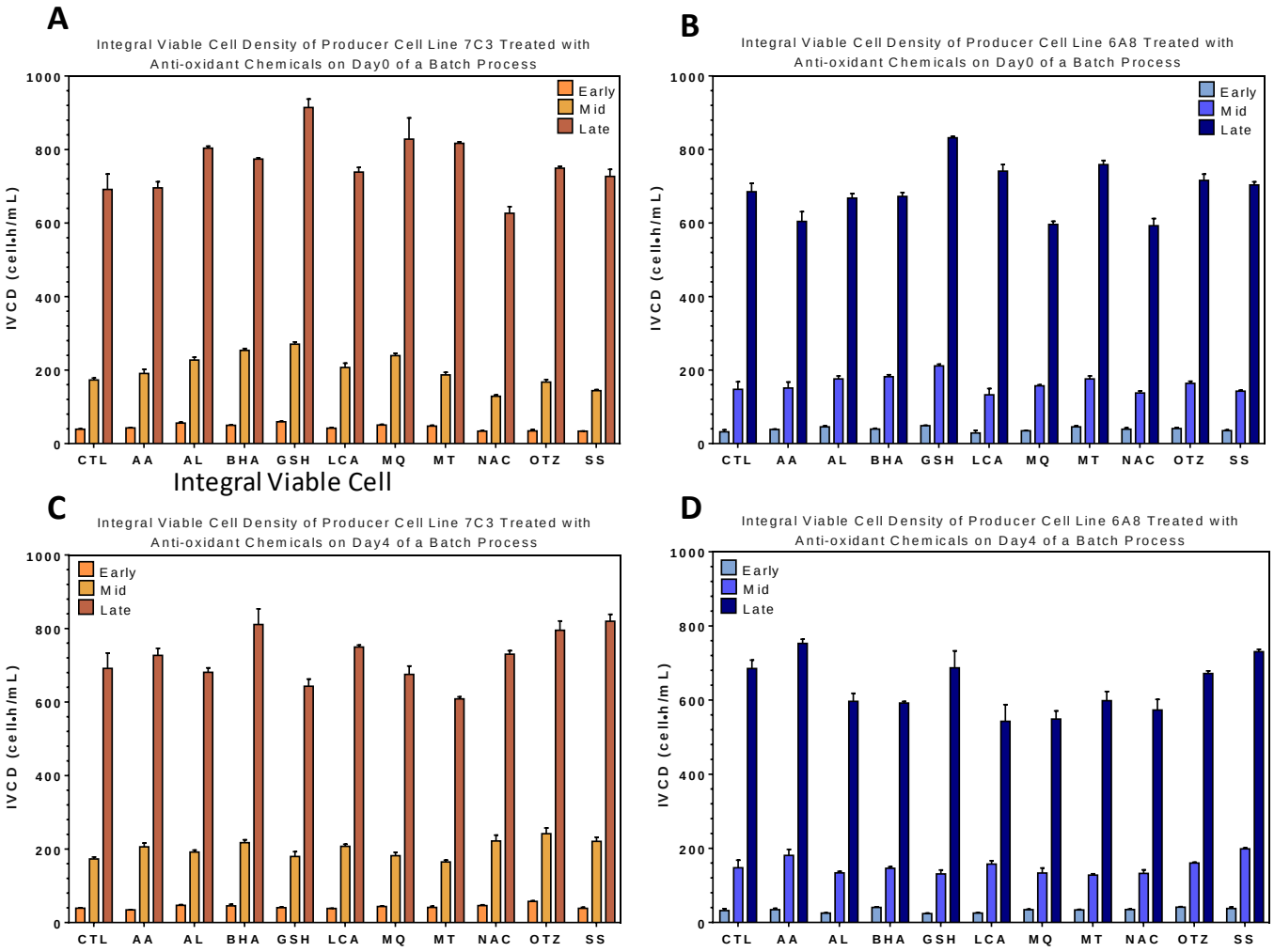

Figure 6a.4. Integral Viable cell Density of 7C3 and 6A8 producer cell lines in a batch culture process when treated with anti-oxidant chemicals Ascorbic Acid (AA), α-Lipoic Acid (AL), Butylated Hydroxyanisole (BHA), Glutathione (GSH), L-Carnosine (LCA), MitoQ (MQ), MitoTEMPOL (MT), N-Acetylcysteine (NAC), Procysteine (OTZ) and Selenium (SS). IVCD values at 72h ("Early"), 120h ("Mid"), and 168h ("Late") of culture are shown. Data bars represent Mean average of samples taken from triplicate cultures and error bars indicate Standard Error of the Mean. (A) IVCD of 7C3 when treated with AO on Day0 of culture. (B) IVCD of 6A8 when treated with AO on Day0 of culture. (C) IVCD of 7C3 when treated with AO on Day4 of culture. (D) IVCD of 6A8 when treated with AO on Day4 of culture.

Density values for producer cultures treated with AO on Day4 are shown in Figure 6.4.A (7C3) and B (6A8). As with treatment on Day0, there was no mid-stationary phase growth difference in any of the Day4 treated 6A8 cultures in comparison to the untreated control cultures. 7C3 cultures, on the other hand, displayed an increase in IVCD when treated on Day4 with OTZ (242 cell•h/mL, $p<0.05$), and NAC (222 cell•h/mL, $p<0.1$). Again as with Day0 treatment, addition of AO at Day4 had a much

more detrimental effect on the IVCD of 6A8 cultures, with more AO chemicals eliciting a decline in IVCD than an increase. The only AO to positively impact IVCD of 6A8 cultures were AA and SS, with IVCD values at 168h of 753 cell•h/mL ($p<0.01$) and 731 cell•h/mL ($p<0.05$), respectively. However, 6A8 cultures treated on Day4 with AL, BHA, LCA, MQ, MT and NAC all showed a decrease in IVCD. Values observed were 597 cell•h/mL, 592 cell•h/mL, 543 cell•h/mL, 549 cell•h/mL, 598 cell•h/mL, and 573 cell•h/mL, respectively ($p<0.01$). In contrast to the 6A8 cultures, the only AO to have a detrimental effect on late-stage 7C3 cultures when introduced on Day4 was MT, lowering IVCD at 168h to 609 cell•h/mL ($p<0.01$). 7C3 cultures treated on Day4 with BHA, LCA, OTZ and SS, however, had significantly increased IVCD and thus late-stage growth, with observed values of 881 cell•h/mL,

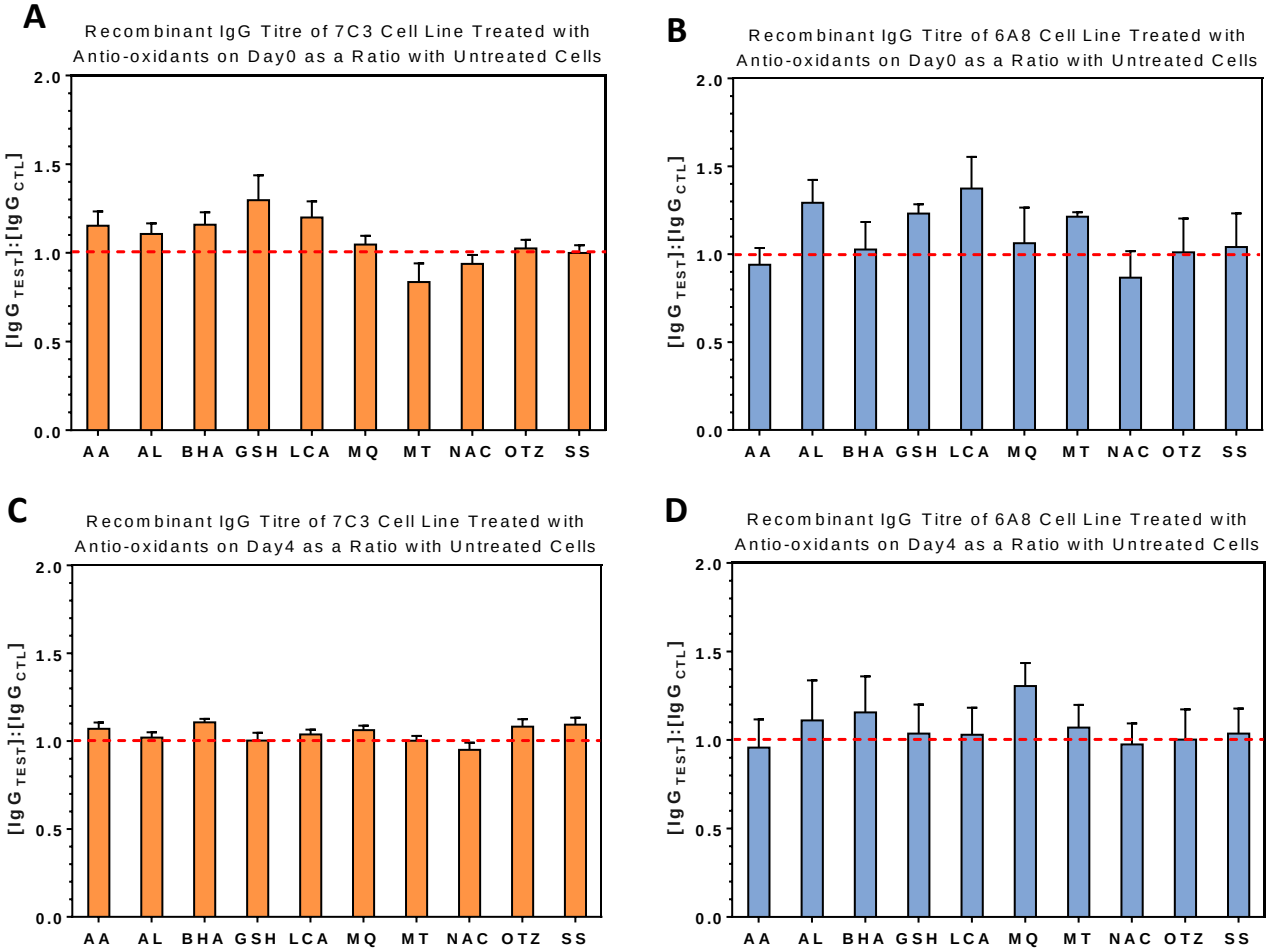

Figure 6a.5. Final Day Titre of 7C3 and 6A8 producer cell lines in a batch culture process when treated with anti-oxidant chemicals Ascorbic Acid (AA), α-Lipoic Acid (AL), Butylated Hydroxyanisole (BHA), Glutathione (GSH), L-Carnosine (LCA), MitoQ (MQ), MitoTEMPOL (MT), N-Acetylcysteine (NAC), Procysteine (OTZ) and Selenium (SS), shown as a ratio with final day titre of untreated cells. Data bars represent Mean average of samples taken from triplicate cultures and error bars indicate Standard Error of the Mean. (A) Final Day Titre ratios of 7C3 when treated with AO on Day0 of culture. (B) Final Day Titre ratios of 6A8 when treated with AO on Day0 of culture. (C) Final Day Titre ratios of 7C3 when treated with AO on Day4 of culture. (D) Final Day Titre ratios of 6A8 when treated with AO on Day4 of culture.

cell•h/mL, 795 cell•h/mL and 820 cell•h/mL, respectively ($p<0.01$ for BHA, OTZ, SS; $p<0.05$ for LCA).

6a.4.3 Impact of Anti-Oxidant Treatment on Productivity

6a.4.3.1 Final Day Product Titre

Final day product titre for cultures treated with AO on Day0 and Day4 of a batch culture process is shown as a ratio with final day titres for untreated cultures in Figure 6a.5. There were clear differences between AO efficacies when introduced at different stages in culture. When comparing the final day titres of 7C3 cultures treated with AO at the point of inoculation (Fig. 6a.5.A), the only impacts on productivity were positive. BHA, GSH and LCA had some impact on product titre, increasing it by 16 %, 30 % and 20 %, respectively ($p<0.1$). Treatment at point of inoculation also saw no negative impact on final day titre for 6A8 cultures (Fig. 6.5.B). As with 7C3, 6A8 cultures treated with GSH on Day0 saw an increase in product titre, in this case by 23 % ($p<0.05$). In contrast to 7C3, BHA and LCA had no impact on 6A8 cultures when introduced on Day0. There were, however, increases in product titre of 29 % and 21 % observed in 6A8 cultures treated on Day0 by AL ($p<0.1$) and MT ($p<0.01$), respectively.

When AO were introduced to cultures at late exponential phase, there were once again no negative impacts on the final day titres of either 7C3 or 6A8 cultures. When AO treated 7C3 cultures were observed, there were only slight increases in productivity seen; in cultures treated with BHA there was an increase of 11 % ($p<0.01$); in cultures treated with MQ there was an increase of 6% ($p<0.1$); and in cultures treated with SS there was an increase of 10 % ($p<0.1$). There was minimal impact on final day titre in 6A8 cultures treated with AO on Day4, with only MQ increasing titre, though this was a substantial 30 % rise ($p<0.1$).

6a.4.3.2 Cell Specific Productivity

The cell specific productivities (q_P) for cultures treated with AO on Day0 and Day4 at 96 h, 144 h and 192 h of a batch culture process are shown in Figure 6.6. Whether AO were added at the point of inoculation or at late exponential phase, there were changes to q_P from the highest number of additives at 96h. In 7C3 cultures most of these effects were negative in relation to the q_P of untreated cultures, however in 6A8 cultures the majority of chemicals elicited a positive change in q_P.

When treated on Day0, there was a reduction in q_P at 96 h in 7C3 cultures treated with AA, BHA and MQ. In comparison to the specific productivity of untreated cultures at this time point, calculated at 2.09 pg/cell•h, these treated cultures displayed a q_P of 1.89 pg/cell•h ($p<0.1$), 1.43 pg/cell•h and 1.52 pg/cell•h (both $p<0.01$), respectively. In contrast, 7C3 cultures treated with NAC and OTZ actually saw an increase in cell specific productivity at the same time point, with calculated

q_P in these cultures standing at 2.49 pg/cell•h and 2.14 pg/cell•h, respectively ($p<0.01$). In 6A8 cultures treated with AO on Day0 of culture, all but two of the chemicals elicited a significant change in specific productivity immediately after introduction of the chemicals, be it positive or negative in comparison to the untreated q_P of 2.02 pg/cell•h. Cultures where a drop in productivity were observed were those treated with AL (0.449 pg/cell•h, $p<0.05$), GSH (0.606 pg/cell•h, $p<0.01$) and MT (0.727 pg/cell•h, $p<0.01$). In contrast, 6A8 cultures with an observed increase in q_P at 96 h were treated on Day0 with AA (2.44 pg/cell•h, $p<0.05$), LCA (3.05 pg/cell•h, $p<0.05$), MQ (2.39 pg/cell•h, $p<0.01$), NAC (2.49 pg/cell•h, $p<0.01$) and SS (2.76 pg/cell•h, $p<0.01$).

As 7C3 cultures progressed to mid-stationary phase, the effects on q_P seen earlier in culture were no longer observed, but other AO introduced on Day0 were now having an impact. There was an observed decrease in q_P at 144 h in 7C3 cultures treated with AL, GSH and MT, with calculated q_P for these cultures standing at 0.187 pg/cell•h, 0.11 pg/cell•h and 0.242 pg/cell•h, respectively

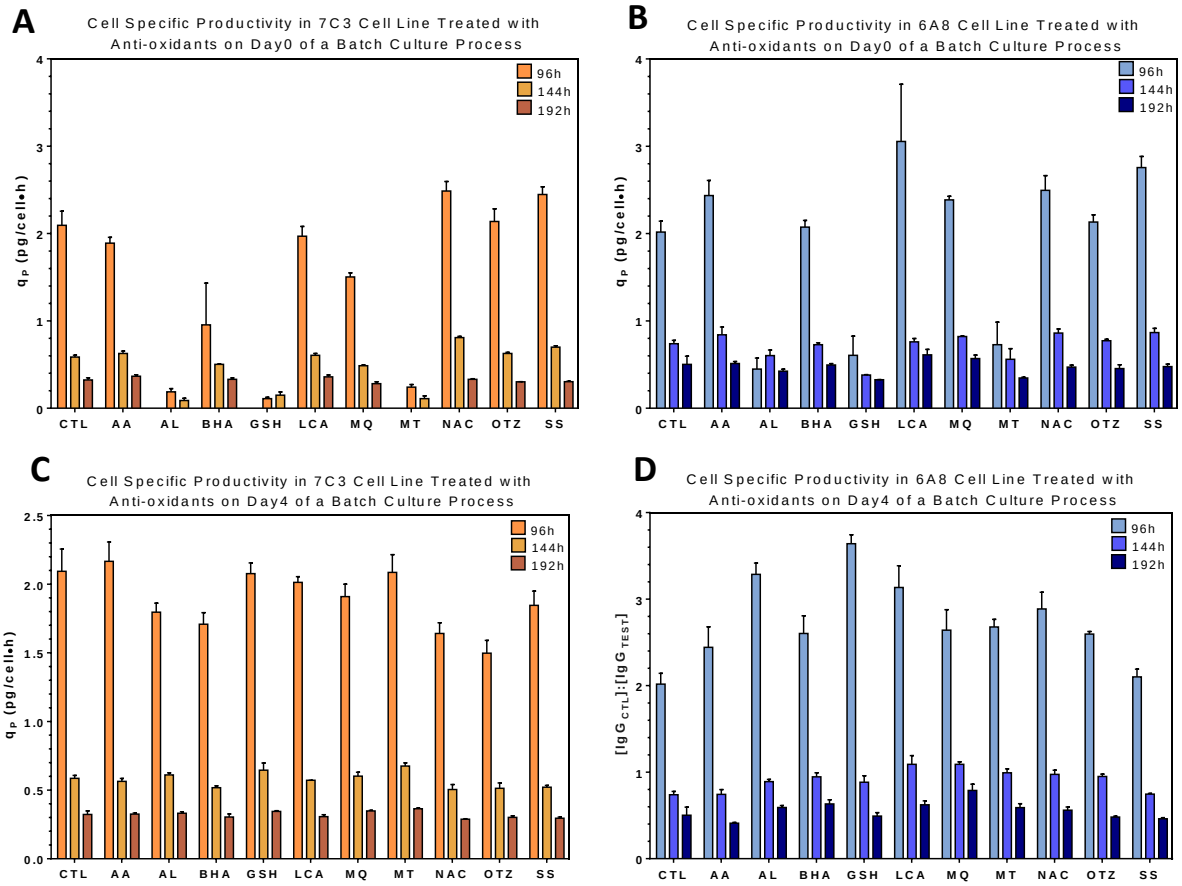

Figure 6a.5. Cell Specific Productivity (q_P) of 7C3 and 6A8 producer cell lines at 96 h, 144 h and 192 h in a batch culture process when treated with anti-oxidant chemicals Ascorbic Acid (AA), α-Lipoic Acid (AL), Butylated Hydroxyanisole (BHA), Glutathione (GSH), L-Carnosine (LCA), MitoQ (MQ), MitoTEMPOL (MT), N-Acetylcysteine (NAC), Procysteine (OTZ) and Selenium (SS). Data bars represent Mean average of samples taken from triplicate cultures and error bars indicate Standard Error of the Mean. (A) q_P of 7C3 when treated with AO on Day0 of culture. (B) q_P of 6A8 when treated with AO on Day0 of culture. (C) q_P of 7C3 when treated with AO on Day4 of culture. (D) q_P of 6A8 when treated with AO on Day4 of culture.

($p<0.01$). This was in comparison to the q_P at 144h of untreated 7C3 cultures, which was calculated at 0.586 pg/cell•h. Only 6A8 cultures treated with GSH on Day0 of culture continued to see any effect on q_P at mid-stationary phase, with an observed decrease from 0.739 pg/cell•h in untreated cultures to 0.381 pg/cell•h ($p<0.05$).

There was no observed impact on cell specific productivity at 192 h in 6A8 cultures treated with AO on Day0. In a continuation from mid-stationary phase, 7C3 cultures treated on Day0 with AL, GSH and MT saw a decrease in q_P at late-stationary phase. The calculated q_P for untreated 7C3 cultures at this time point was 0.323 pg/cell•h. In comparison, the calculated q_P for those treated with AL, GSH and MT was 0.0592 pg/cell•h, 0.15 pg/cell•h and 0.11 pg/cell•h, respectively ($p<0.01$).

In 7C3 cultures treated with AO at late-exponential phase, there was only an impact on cell specific productivity at 96 h, or immediately after the addition of AO. There was no lasting impact, however, with 7C3 treated cells at 144 h and 92 h showing no significant difference to the untreated cultures. Cultures that did show a change in q_P at 96 h were those treated with AL, BHA, NAC, OTZ and SS. All such cultures displayed a reduced q_P in comparison to untreated 7C3 cultures, with calculated values of 1.8 pg/cell•h, 1.71 pg/cell•h, 1.64 pg/cell•h, 1.5 pg/cell•h and 1.85 pg/cell•h ($p<0.05$ for AL and SS, $p<0.01$ for the others). All but one (SS) of the AO added to 6A8 cultures at Day4 had a positive impact on q_P at 96 h (i.e. immediately after addition of the AO). The greatest increase in q_P was seen in cultures treated with GSH, AL and LCA, with calculated q_P values of 3.64 pg/cell•h, 3.29 pg/cell•h and 3.13 pg/cell•h, respectively ($p<0.01$). NAC, MT and MQ showed increases in the middle range, with q_P values for cultures treated with these AO calculated at 2.89 pg/cell•h, 2.67 pg/cell•h and 2.64 pg/cell•h, respectively ($p<0.01$). 6A8 cultures treated with BHA, OTZ and AA on Day4 showed the smallest immediate increase in qP, with calculated values of 2.6 pg/cell•h ($p<0.01$), 2.6 pg/cell•h ($p<0.01$) and 2.44 pg/cell•h ($p<0.05$), respectively. There was no observable difference in the q_P of 6A8 cultures treated with SS at this time point, or indeed at any point in culture.

6A8 cultures treated at late-exponential phase with LCA, MQ, MT and OTZ continued to display an increase in q_P in comparison to untreated cultures. The q_P values calculated for such cultures were 1.09 pg/cell•h, 1.09 pg/cell•h, 0.99 pg/cell•h and 0.95 pg/cell•h, respectively ($p<0.1$ for LCA and MQ, $p<0.05$ for MT and OTZ). There were no observed changes in q_P when either 7C3 or 6A8 were treated on Day4 with any anti-oxidant chemical.

Of the cultures that saw increases in final day product titre when treated with anti-oxidants on either Day0 or Day4, there were no correlating increases in q_P. 7C3 cultures treated on Day0 with BHA, GSH and LCA had an increased final day titre, but q_P was actually decreased in the late stages of GSH treated cultures, LCA had no observable difference in q_P and the only difference in q_P of BHA

treated cultures was a decrease in the earlier stages of growth. Similarly, 6A8 cultures treated on Day0 with AL, GSH and MT saw a rise in final day titre, but only GSH had an impact on q_P in the later stages of growth and this was negative. AL and MT treated cultures saw decreased q_P in the earlier stages of growth, but there was no impact later on. In 7C3 cultures treated on Day4, there were product titre increases in cultures treated with BHA, MQ and SS, but only BHA had any impact on q_P, and that was to decrease it in the earlier stages of growth. These correlations, or lack thereof, indicate that the increased product titre was due to increased growth of these cultures, not because of an increase in specific productivity. The only exception to these patterns was in 6A8 cultures treated with MQ on Day4, in which there was an increased cell specific productivity at 96 h and 144 h, and then an increased final day product titre. This suggests that treatment of 6A8 cultures at late exponential phase of growth is able to increase productivity on a cellular level.

Chapter Summary

Producer cell lines 7C3 and 6A8 were cultured in a batch process and treated at either Day0 or Day4 of culture with chemicals designed to either negatively (pro-oxidant) or positively (anti-oxidant) impact the redox metabolism of the cells. Responses to oxidative perturbation in a batch process appeared to be cell line specific; despite the similarities between 7C3 and 6A8 cell lines, they saw large differences in their responses to anti-oxidants in particular, suggesting that the nature of their oxidative metabolism is different.

Culturing cells in a fed-batch process is more relevant to an industrial process and so data from these batch experiments will be used to engineer novel feeding strategies to encourage oxidative stress resistance and improve growth and productivity characteristics.

Chapter 6b:

Anti-Oxidant Supplementation in Fed-Batch Culture Processes

Contents

List of Abbreviations

CHO	Chinese Hamster Ovary	LCA	L-Carnosine
DoE	Design of Experiments	GSH	Glutathione
IVCD/$IVCD_{MAX}$	Integral Viable Cell Density / Maximal Integral Viable Cell Density	MT	MitoTEMPOL
ROS	Reactive Oxygen Species	AL	α-Lipoic Acid
[rP]	Recombinant Protein Concentration	SS	Sodium Selenite
q_P	Cell Specific Productivity	NAC	N-Acetylcysteine
RFU	Relative Fluorescence Units		

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6b.1 Introduction

The initial screening of anti-oxidant supplementation of industrially relevant producer CHO cell lines in a batch process provides important insights into the oxidative metabolism of these cell lines. However, utilising anti-oxidants in a novel feeding strategy for fed-batch processes is likely to be more relevant in terms of process development in an industrial setting. Design of Experiments is a powerful tool often used in industry to streamline the development process, and so was utilised here in an attempt to predict appropriate anti-oxidant combinations for feeding strategies to improve the growth, productivity and oxidative metabolism of producer cell lines 7C3, 6A8 and APC. Further screening was also conducted based on the data from these initial fed-batch trials in an attempt to engineer a feeding strategy that could benefit all three cell lines, and thus move away from a cell line specific strategy, which ultimately would be more costly and time-consuming to implement for each new cell line in an industrial cell line and process development scheme.

6b.2 Experimentation

Design of Experiments analysis was conducted according to Chapter 3 "*Materials and Methods*", Section 3.4.5 "*Design of Experiments*", pg44. Producer cell lines 7C3, 6A8 and APC were cultured in a fed-batch process according to 3.4.1 "*Culture Conditions*", pg40, and productivity was determined according to 3.4.2 "*Productivity*", pg43. Anti-oxidants were added to basal media and feeding media according to Table 3.3 and 3.5, pg 42 and 43. Cell samples were stained, fixed and analysed using a FACSAria II Flow Cytometer (Beckman Coulter, Tewksbury, US) as described in 3.4.3 "*Reactive Oxygen Species Quantification*", pg43.

6b.3 Design of Experiments

Design of Experiments (DoE) was used to compare the final day product titre, final day cell specific productivity, $IVCD_{MAX}$ and final day Reactive Oxygen Species (ROS) content of 6A8 and 7C3 cell lines when treated with an anti-oxidant either on Day0 or Day4 of culture, and to generate a model to predict the impact of anti-oxidant feeding. All anti-oxidants trialled were analysed separately, and it was possible to elucidate whether 7C3 or 6A8 would have a predicted larger response to the chemical, and whether or not there was a predicted larger response to the addition of chemicals on Day0 or on Day4.

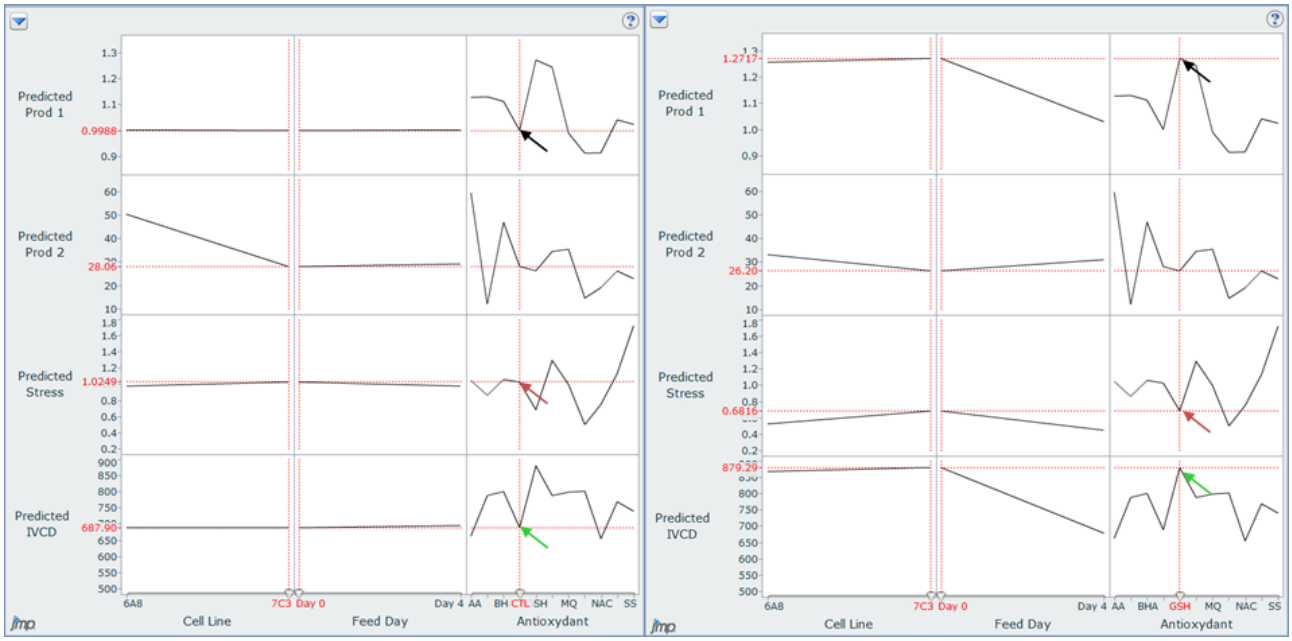

Figure 6b.1. Example histograms generated by Design of Experiments model. Histogram show Top to Bottom predicted product titre ratio, predicted cell specific productivity, predicted ROS level ratios and predicted $IVCD_{MAX}$; Left to Right are shown Cell Line, Feed Day and Anti-oxidant. (A) The predicted values for a 7C3 culture untreated on Day0. (B) The predicted values for a 7C3 culture treated with Glutathione on Day0. Final day product titre is predicted to be 26 % higher than that of the untreated culture (black arrows), and predicted $IVCD_{MAX}$ is predicted to increase by nearly 200 cell•h/mL (green arrows). In contrast, reactive oxygen species levels were predicted to decrease by 32 % in contrast to the untreated cultures (red arrows).

The statistical model created by Gwen Ninon (FUJIFILM UK, Process Design Group) was fed into software containing interactive histograms in order to facilitate comparison of parameters. Screenshots of these histograms accounting for each parameter are contained in Appendix 6, but an example comparison of histograms for untreated cultures and treated cultures is shown in Fig.6b.1.

Based on the predicted values of each parameter at both feeding times, feeding strategies were selected for 7C3 and 6A8. Predictions that suggested a statistically significant ($p<0.05$) change were given precedence, but were not used exclusively in feeding combination determination. These strategies were not identical, due to the differing responses to anti-oxidant chemicals seen in batch cultures of each cell line. For a more general assessment of the applicability of feeding regimes a third cell line, APC, was introduced. Since this cell line had not been trialled in a batch system, a selection of combinations from the other two cell lines were trialled.

6b.4 Fed-batch Anti-Oxidant Feeding Strategy: Trial 1

6b.4.1 Effect of Anti-Oxidant Feeding on Growth of Producer Cell Lines

Table 6b.1. Fed-Batch Experimentation Trial 1. The trialled feeding schemes are detailed with the anti-oxidants used, their concentrations and the relevant timings.

CODE	CELL LINE	BASAL SUPPLEMENT	CONCENTRATION	FEED SUPPLEMENT	CONCENTRATION
A	7C3	Glutathione	50 µM	MitoTEMPO	5 nM
B	7C3	L-Carnosine	10 mM	MitoTEMPO	5 nM
C	7C3	L-Carnosine	10 mM	α-Lipoic Acid	25 µM
D	7C3	L-Carnosine + Glutathione	10 mM/50 µM	MitoTEMPO	5 nM
E	7C3	L-Carnosine + Glutathione	10 mM/50 µM	--	N/A
F	7C3	Ascorbic Acid	100 µM	N-Acetylcysteine	1 mM
G	6A8	Glutathione	50 µM	Butylated Hydroxyanisole	1 µM
H	6A8	L-Carnosine + Glutathione	10 mM/50 µM	--	N/A
I	6A8	α-Lipoic Acid	25 µM	Sodium Selenite	100 nM
J	6A8	L-Carnosine	10 mM	MitoQ	10 nM
K	6A8	L-Carnosine	10 mM	α-Lipoic Acid	25 µM
L	6A8	Glutathione + MitoTEMPO	50 µM/5 nM	--	N/A
M	APC	Glutathione + MitoTEMPO	50 µM/5 nM	--	N/A
N	APC	L-Carnosine + Glutathione	10 mM/50 µM	--	N/A
O	APC	α-Lipoic Acid	25 µM	Sodium Selenite	100 nM
P	APC	Ascorbic Acid	100 µM	N-Acetylcysteine	1 mM
Q	APC	L-Carnosine	10 mM	α-Lipoic Acid	25 µM
R	APC	L-Carnosine	10 mM	MitoTEMPO	5 nM
S	APC	L-Carnosine	10 mM	MitoQ	10 nM
T	APC	Glutathione	50 µM	MitoQ	10 nM
U	APC	Glutathione	50 µM	MitoTEMPO	5 nM
CTL	ALL	--	N/A	--	N/A

The Integral Viable Cell Density (IVCD) or producer cell lines 7C3, 6A8 and APC in a fed-batch process when treated with anti-oxidant feeding strategies are shown in Fig.6b.2. By mid-exponential phase of growth ("Early"), no feeding strategies trialled had any significant impact on the growth of these three cell lines. During stationary phase ("Mid", Fig. 6b.2.A), producer cell line 7C3 was largely

unaffected, and in fact strategies B and C ($p<0.1$), and strategy F ($p<0.01$) had a negative impact on cell growth, with IVCD values of 396 cell•h/mL, 378 cell•h/mL and 361 cell•h/mL, respectively (compared to the untreated value of 412 cell•h/mL). In contrast, by the time the cell cultures reached late stationary phase ("Late", Fig. 6b.2.A)), all feeding strategies had significantly improved IVCD in comparison to the untreated culture IVCD of 727 cell•h/mL. The highest IVCD values were seen from feeding strategies E, D, F, and B with values of 985 cell•h/mL, 970 cell•h/mL, 965 cell•h/mL and 964 cell•h/mL, respectively. Feeding strategies C and A had IVCD values of 860 cell•h/mL and 848 cell•h/mL, still more than 100 cell•h/mL higher than the untreated cells at this stage in culture growth.

During stationary phase ("Mid", Fig.6b.2.B), only feeding strategy G had a positive impact on IVCD of 6A8 cultures, with an increased value of 448 cell•h/mL in comparison to the untreated IVCD at this time, of 395 cell•h/mL ($p<0.05$). Feeding strategies K and I decreased IVCD at this time point, with observed values of 343 cell•h/mL and 294 cell•h/mL, respectively ($p<0.05$). Feeding strategies H, J and L continued to exert no influence on IVCD at this time point, as with measurements made earlier. As the cells progressed into late stationary phase ("Late", Fig. 6b.2.B), all feeding strategies had a significant impact on the IVCD of treated cultures in comparison to that of the 1210 cell•h/mL seen in untreated cultures. However, it was still only feeding strategy G that made a positive impact, increasing IVCD to 1308 cell•h/mL. Feeding strategies J, H and K had the largest negative impact on IVCD, with observed IVCD values of 920 cell•h/mL, 966 cell•h/mL and 980 cell•h/mL, respectively. Observed IVCD of cultures treated by strategies L and I were 1102 cell•h/mL and 1006 cell•h/mL, respectively.

When observing the IVCD of treated APC cultures, it can be seen that there is a stark difference between treated cell growth during early stationary phase ("Mid", Fig.6b.2.C) and during late stationary phase ("Late", Fig.6b.2.C). At the earlier time point, the majority of feeding strategies that had a significant impact on IVCD did so to a detrimental effect. However, as the cells progressed through culture to the latter stages, almost all feeding strategies had a positive impact on IVCD. At 168h in culture, untreated APC cultures had an observed IVCD of 487 cell•h/mL, with cultures treated using strategy M having an increased IVCD of 550 cell•h/mL ($p<0.01$). Feeding strategies R, N, S and O, in contrast, had decreased observed IVCD values of 431 cell•h/mL, 417 cell•h/mL, 405 cell•h/mL and 389 cell•h/mL, respectively.

In the majority of cases, feeding strategies were able to extend the culture period of APC cell cultures, with feeding strategies N, O, P, Q, R and S all lasting two more days than the untreated cultures. As a result, these cultures displayed substantially increased IVCD values at late stationary phase of 1154 cell•h/mL, 1137 cell•h/mL, 1340 cell•h/mL, 1186 cell•h/mL and 1126 cell•h/mL,

respectively ($p<0.01$), compared to the untreated culture IVCD value of 869 cell•h/mL. Cultures treated with feeding strategies M, T and U did not have extended culture periods, although feeding strategy M maintained an increased growth at this stage, with an observed IVCD of 948 cell•h/mL ($p<0.01$). Feeding strategy U did not have an impact on late stage IVCD, and strategy T displayed a decreased IVCD in comparison to untreated cultures, with an IVCD of 790 cell•h/mL ($p<0.01$).

6b.4.2 Effect of Anti-Oxidant Feeding on Productivity of Producer Cell Lines

Figure 6b.3 indicates the recombinant product titre ([rP]) of 7C3, 6A8 and APC cell lines in a fed-batch process when treated with anti-oxidant chemicals. As the lowest producer of the three, [rP] for 7C3 was too low at the "Early" time point (120h) to be detected using the ValitaTITER assay. Values are shown, however, for the "Mid" and "Late" time points of 168 h and 240 h. In the cases of 6A8 and APC cell lines, data was obtained for all three time points. It is clear from the data that there was less of an impact on product titre when treated with anti-oxidants than there was on IVCD.

There was no significant impact on product titre of 7C3 cultures at 168 h when treated with any of the anti-oxidant feeding regimes (Fig.6b.3.A). At 240 h, however, feeding strategies B, D and E had an observed increase in [rP], with concentrations of 366.32 µg/mL ($p<0.05$), 379.06 µg/mL and 375.07 µg/mL ($p<0.01$), respectively, in comparison with the untreated [rP] at this time point of 295.52 µg/mL. Feeding strategy F, however, had a negative impact on productivity, with an observed [rP] at 240 h of 193.91 µg/mL. Despite an overall increased productivity in comparison with its sister cell line, there were no observed differences in [rP] in any of the 6A8 cultures at any time point, treated or otherwise (Fig.6b.3.B).

As with 7C3, there were no observed differences in product titre in APC cultures at 168 h; however, various feeding strategies had an impact on [rP] at 240 h (Fig.6b.3.C). Feeding strategies O and R were able to increase [rP] at this time point ($p<0.05$), with concentrations of 2181.04 µg/mL and 2095.46 µg/mL, respectively, in comparison to the untreated [rP] of 1951.07 µg/mL. An increased level of growth in cell lines treated with feeding strategy Q, there was a decreased level of productivity, with [rP] at 240 h measuring 1808.26 µg/mL ($p<0.05$). There was also a decreased [rP] in cultures treated with feeding strategies U and T, with observed product titres at this time point of 1809.92 µg/mL ($p<0.05$) and 1483.01 µg/mL ($p<0.01$), respectively.

There were no significant differences observed in the cell specific productivity (q_P) of 6A8 cultures when treated with any of the feeding strategies trialled (Fig. 6b.4.B), nor were there any differences in 7C3 cultures at 168 h of their culture period (Fig 6b.4.A) – a result to be expected since there were no observed differences in either IVCD nor product titre at this time point. Later in culture, however, the effect on the combined IVCD and [rP] led to there being a significant impact on

cell specific productivity at 240 h when utilising all feeding strategies trialled. Feeding strategy A was the only one to elicit a positive change on q_P, with a calculated rate of 0.655 pg/cell•h compared with the untreated culture q_P at the same time point of 0.540 pg/cell•h ($p<0.05$). Despite some increases in late stage [rP], correlating increases in IVCD actually meant that all other feeding strategies had a decreased calculated q_P at late stationary phase of growth ($p<0.01$). The largest decreases in q_P correlated with the second highest increase in IVCD, being seen in cultures treated with feeding strategy F (0.178 pg/cell•h). Other feeding strategies C, D, E and B saw decreased q_P values of 0.270 pg/cell•h, 0.359 pg/cell•h, 0.361 pg/cell•h and 0.368 pg/cell•h, respectively.

Largely (but not wholly) due to the substantial increases in IVCD at late stage culture in comparison to untreated cultures, all changes to q_P in APC cultures at 240 h were negative (Fig. 6b.4.C). The closest q_P to the untreated cultures of 3.07 pg/cell•h were those treated with feeding strategies T and M, which showed a calculated q_P of 2.54 pg/cell•h ($p<0.1$) and 2.42 pg/cell•h ($p<0.05$), respectively. Feeding strategies N, Q, R and S were much lower, with calculated q_P values of 1.85 pg/cell•h, 1.48 pg/cell•h, 1.77 pg/cell•h and 1.86 pg/cell•h, respectively ($p<0.01$). As with 6A8 and 7C3 cultures, there were no significant differences in q_P for any treated APC cultures at earlier stages of culture.

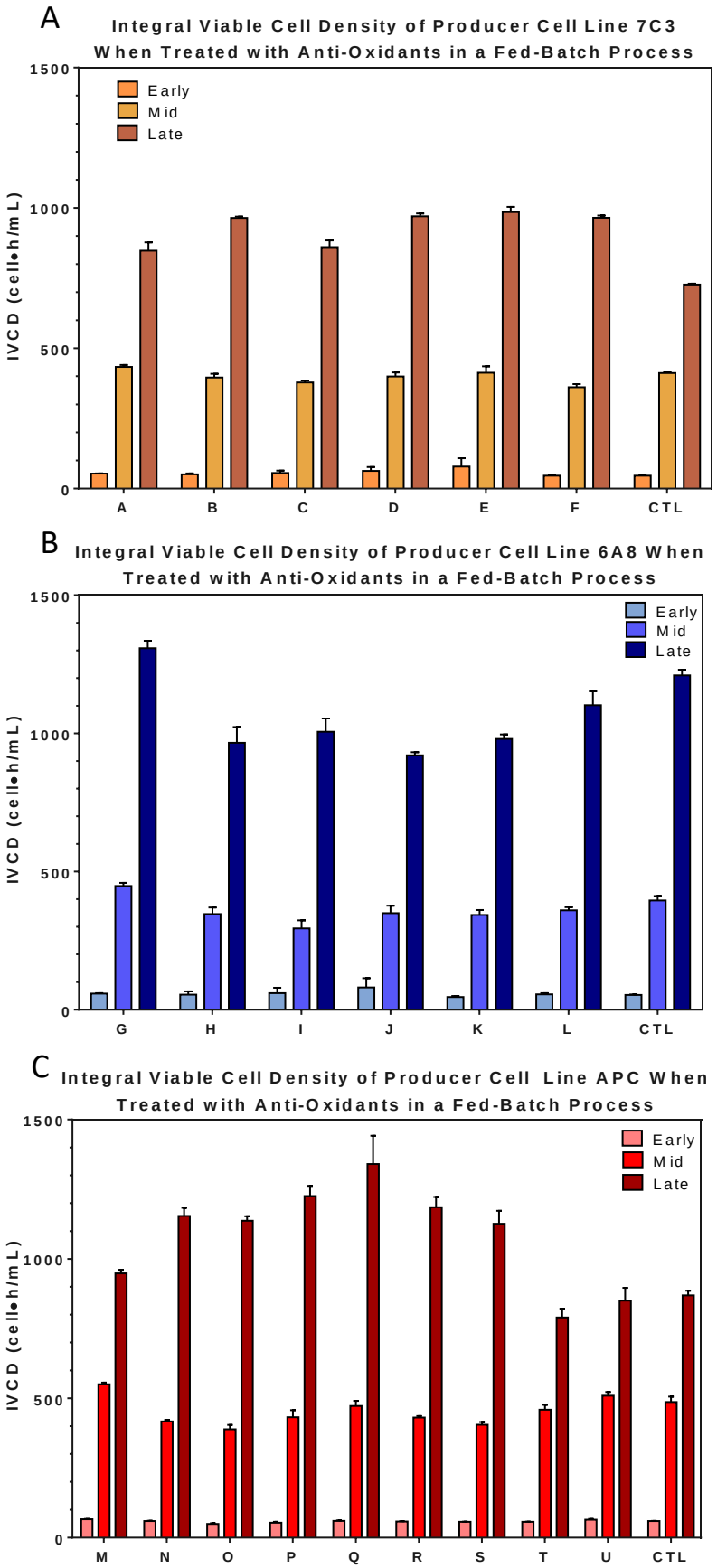

Figure 6b.2. (A) Integral Viable Cell Density at 96 h ("Early"), 168 h ("Mid") and 264 h ("Late") of 7C3 cultures treated with anti-oxidants in a fed-batch culture. (B) Integral Viable Cell Density at 96 h ("Early"), 168 h ("Mid"), and 288 h ("Late") of 6A8 cultures treated with anti-oxidants in a fed-batch culture. (C) Integral Viable Cell Density at 9 6h ("Early"), 168 h ("Mid"), and 288 h ("Late") of APC cultures in a fed-batch process. ["Late" bars show IVCD at 240 h for M, T, U and CTL]. Data bars indicate the Mean Average of triplicate cultures, with error bars indicating Standard Error of the Mean.

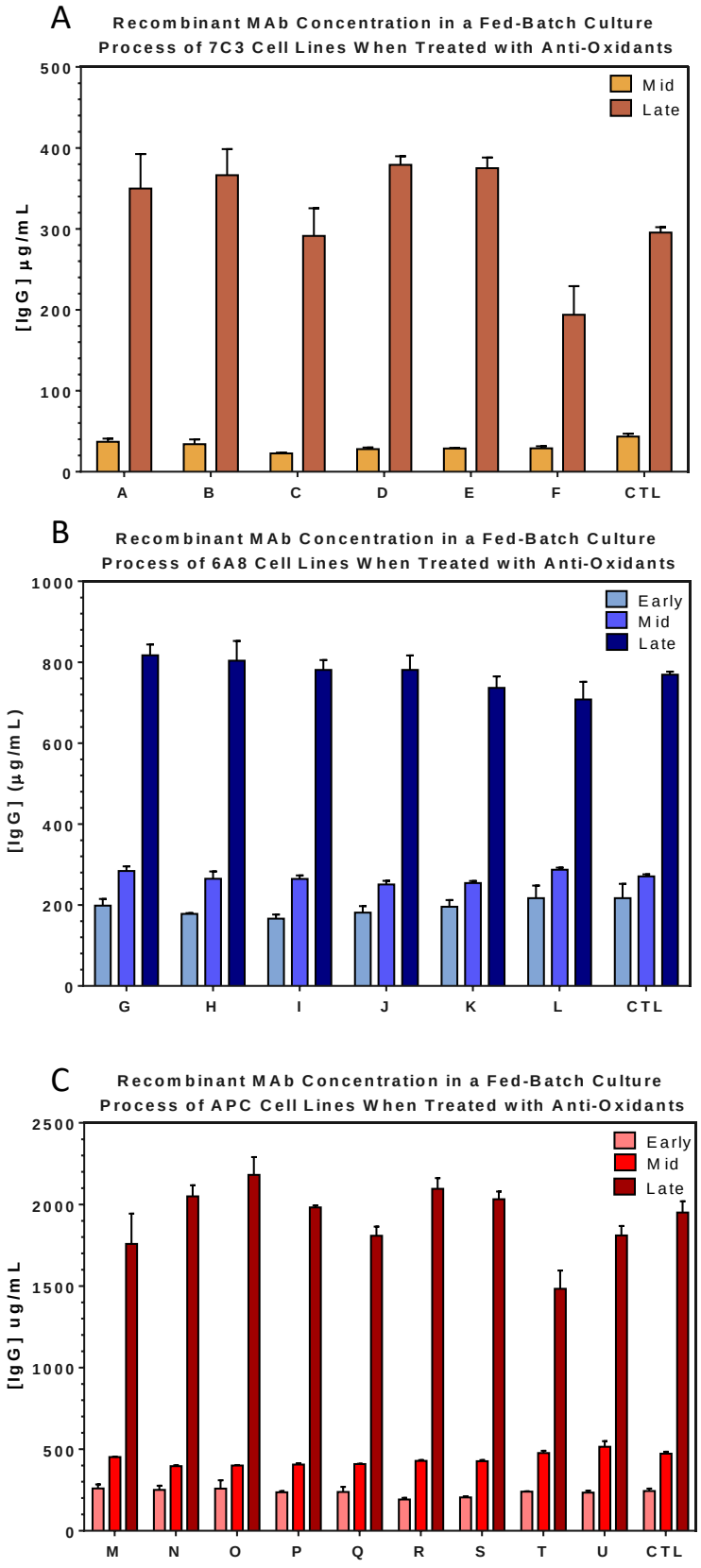

Figure 6b.3. (A) Recombinant product titre of 7C3 cultures at 168 h ("Mid") and 240 h (Late") in a fed-batch process when treated with anti-oxidants. (B) Recombinant product titre of 6A8 cultures at 120 h ("Early"), 168 h ("Mid") and 240 h ("Late") in a fed-batch process when treated with anti-oxidants. (C) Recombinant product titre of APC cultures at 120 h ("Early"), 168 h ("Mid") and 240 h ("Late") in a fed-batch process when treated with anti-oxidants. Data bars show Mean Average of triplicate cultures, with error bars indicating Standard Error of the Mean.

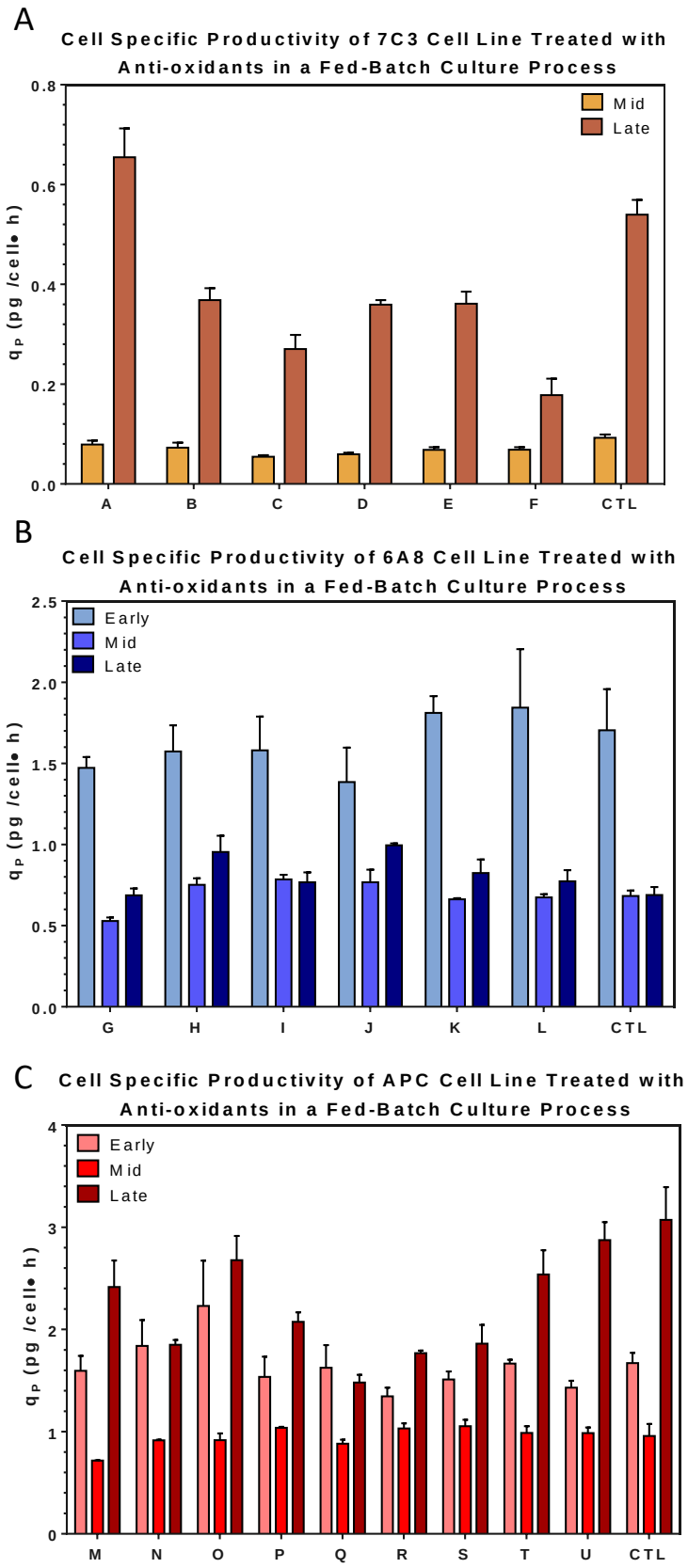

Figure 6b.4. (A) Cell Specific Productivity of 7C3 cultures at 168 h ("Mid") and 240 h (Late") in a fed-batch process when treated with anti-oxidants. (B) Cell Specific Productivity of 6A8 cultures at 120 h ("Early"), 168 h ("Mid") and 240 h ("Late") in a fed-batch process when treated with anti-oxidants. (C) Cell Specific Productivity of APC cultures at 120 h ("Early"), 168 h ("Mid") and 240 h ("Late") in a fed-batch process when treated with anti-oxidants. Data bars show Mean Average of triplicate cultures, with error bars indicating Standard Error of the Mean.

6b.4.3 Effect of Anti-Oxidant Feeding on Reactive Oxygen Species Levels of Producer Cell Lines

The decrease in q_P seen in 7C3 cultures correlated with an increase in Reactive Oxygen Species levels in the latter stages of culture (Fig. 6b.5.A). In particular, at 264 h of culture ("DAY11"), cultures treated with feeding strategies B, C and E had fluorescence intensities of 11374 RFU ($p<0.1$), 12274 RFU ($p<0.05$) and 18963 RFU ($p<0.01$), respectively, in contrast to the untreated culture fluorescence intensity of 8566 RFU. Feeding strategies also elicited an increase in ROS levels earlier in culture, with 7C3 cells treated with feeding strategies A, B, D and E showing fluorescence intensity readings at 144 h ("DAY6") of 16484 RFU ($p<0.05$), 5612.5 RFU ($p<0.05$), 5360 RFU ($p<0.1$) and 8068 RFU ($p<0.05$) respectively, in contrast to the untreated culture reading of 2031 RFU.

6A8 cultures, in contrast, saw very little change in their ROS levels when treated with anti-oxidant feeding strategies (Fig 6b.5.B). Cultures treated with feeding strategies G and I, however, did see a reduction in ROS levels at 264 h, with CellROX fluorescence intensities of 3915 RFU ($p<0.01$) and 4489 RFU ($p<0.05$), respectively, compared to the fluorescence intensities of untreated cells of 6321 RFU. This decrease in ROS levels correlates with an increase in IVCD for cells treated with feeding strategy G.

The feeding strategies employed for APC cell lines had a more beneficial impact on ROS levels earlier in the stationary phase of growth (Fig. 6b.5.C). Lower fluorescence intensities were observed in cultures treated with feeding strategies O, P, R and U, with observed readings of 3320 RFU ($p<0.01$), 3766 RFU ($p<0.05$), 3360 RFU ($p<0.01$) and 3342 RFU ($p<0.05$), respectively, compared to the untreated culture readings of 4746 RFU. However, as the cells progressed to later stages in culture, the decrease in ROS was not maintained. In contrast to the fluorescence intensity in untreated cultures of 4098 RFU, feeding strategies N, O, P, R and S elicited fluorescence intensities of 5360 RFU ($p<0.1$), 5929 RFU ($p<0.01$), 5091 RFU ($p<0.01$), 5639 RFU ($p<0.05$) and 4106 RFU ($p<0.1$), respectively.

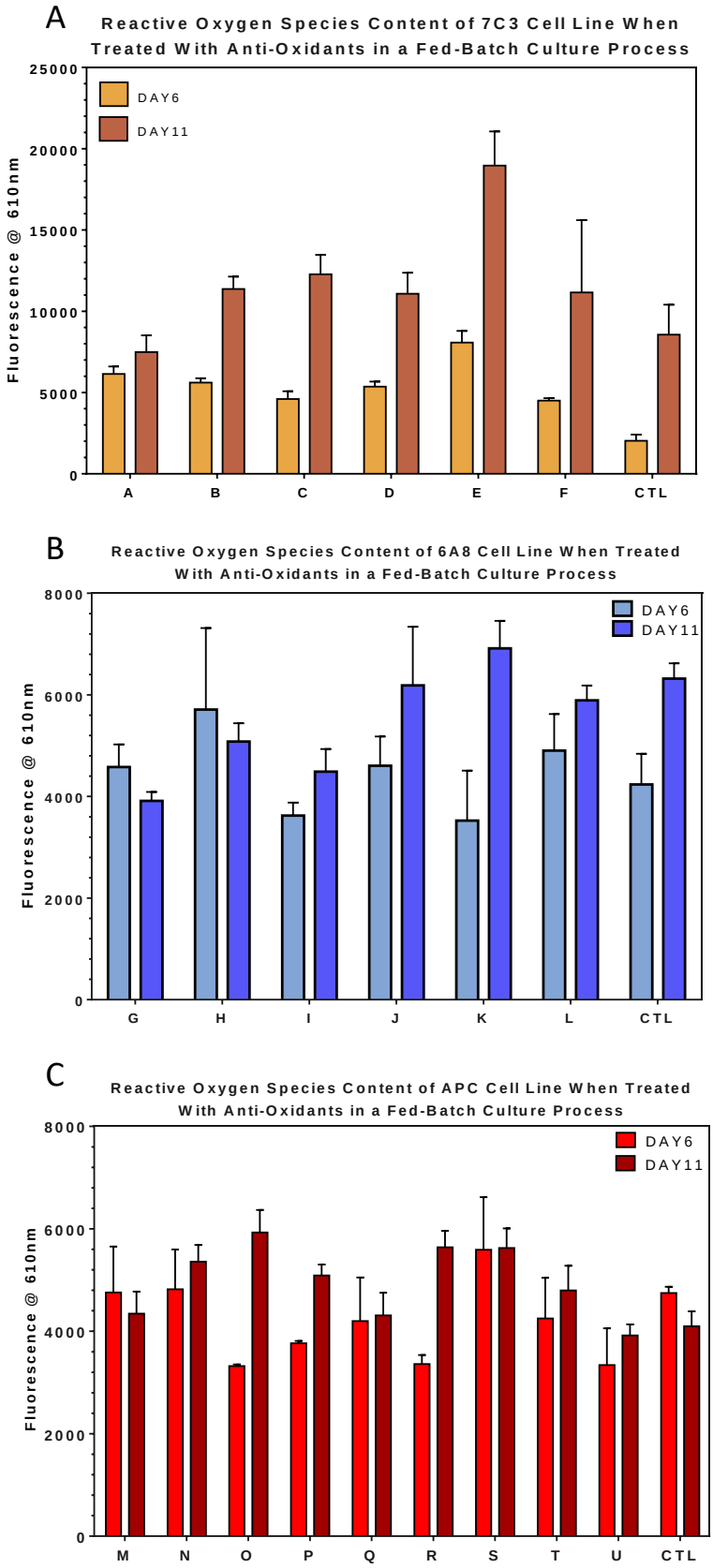

Figure 6b.5. (A) CellROX Fluorescence Intensity at 610 nm of 7C3 cultures at 144 h ("DAY6") and 264 h ("DAY11") in a fed-batch process when treated with anti-oxidants. (B) CellROX Fluorescence Intensity at 610 nm of 6A8 cultures at 144 h ("DAY6") and 264 h ("DAY11") in a fed-batch process when treated with anti-oxidants. (C) CellROX Fluorescence Intensity at 610 nm of APC cultures at 144 h ("DAY6") and 264 h ("DAY11") in a fed-batch process when treated with anti-oxidants. Data bars show Mean Average of triplicate cultures, with error bars indicating Standard Error of the Mean.

6b.4.4 Feeding Strategy Determination for Trial 2

As with the batch culture trials, there were clear trends seen across the three cell lines in response to certain anti-oxidant chemicals, despite varying feeding strategies being employed between cell lines. For example, many cultures with L-carnosine (LCA) included in the basal media saw improved performance phenotypes; feeding strategies B, C, D and E in 7C3 cultures and N, Q, R and S in APC cultures all contained LCA in the basal media and all saw significant increases in $IVCD_{MAX}$. Of these cultures, cells treated with feeding strategies B, D, E, R and S also saw significant increases in final day product titre, in comparison with the untreated cells.

Another anti-oxidant chemical that performed well when included in the basal media was Glutathione (GSH), with feeding strategies D, E, and N all including GSH at the beginning of culture and all having increased observed $IVCD_{MAX}$ and final day titre. Incidentally, these all were feeding strategies where LCA and GSH were combined in the basal media, and neither feeding strategy E nor N had any additional anti-oxidant included in the feeding media. When observing additives included in the feeding media, MitoTEMPOL (MT) stood out as having the most positive impact on the trialled cell lines; feeding strategies A, B, D and R all featured MT in the feeding media, and all had improved growth, product titre, or both. Feeding strategy U was the only one trialled with MT in the feed that did not display any improved characteristics.

Whilst none of the 6A8 cultures trialled saw an increase in growth or productivity, they were the only cultures in which a decrease in ROS levels in the later stages of culture was observed. Feeding strategies G and H, which included GSH and MT or GSH and LCA in the basal media, respectively, saw late stage ROS decrease. Additionally, feeding strategy I also saw a decrease in late-stage intracellular ROS levels. Feeding strategy I featured α-lipoic acid (AL) in the basal media and Sodium Selenite (SS) in the feeding media, a combination not trialled with the other two cell lines. Feeding strategy P saw an overall increase in IVCD, with an anti-oxidant combination of Ascorbic Acid (AA) in the basal media and N-Acetylcysteine (NAC) in the feeding media. Whilst this combination was trialled in 7C3 cultures (feeding strategy F), there was no observed improvement in these cultures.

Due to these observed similarities, the decision was made to move towards identifying an anti-oxidant supplementation strategy that could have a positive impact on the growth, productivity and/or ROS levels of all three cell lines, rather than a cell line specific strategy. There is a continuing industry focus towards timeline reduction and speed-to-clinic, and so it is important to select cell lines that fit a defined process, rather than developing a bespoke process for a selected cell line. The feeding strategies to be trialled focussed heavily on LCA, GSH and MT due to cell line characteristics being repeatedly improved when using them. However, those strategies which perhaps had not

been trialled in all three cell lines but had shown promise in one were also selected for screening. Finally, Glutathione in the basal media during batch-culture elicited very positive results in terms of growth and ROS levels, but was not trialled on its own in this initial fed-batch screen. Thus, in the second fed-batch trial, glutathione was included in the basal media as a feeding strategy without addition of another anti-oxidant, either in the basal media or the feed.

6b.5 Fed-batch Anti-Oxidant Feeding Strategy: Trial 2

Producer cell lines 7C3, 6A8 and APC were all cultured in a fed-batch process and treated with the same feeding strategies, detailed in Chapter 3 "*Materials and Methods*", Table 3.4 "Anti-oxidant Supplementation Protocol 2", pg43 and summarised again below in Table 6b.2.

Table 6b.2. Fed-Batch Experimentation Anti-Oxidant Feeding Trial 2.

CODE	TOTAL FEED SUPPLEMENT	CONCENTRATION	LATE STAGE FEED SUPPLEMENT	CONCENTRATION
α	L-Carnosine	10 mM	MitoTEMPO	5 nM
β	L-Carnosine	10 mM	α-Lipoic Acid	25 µM
γ	Glutathione	50 µM	MitoTEMPO	5 nM
δ	Glutathione	50 µM	--	N/A
ε	α-Lipoic Acid	25 µM	Sodium Selenite	100 nM
ζ	MitoTEMPO	5 nM	--	N/A
η	Ascorbic Acid	100 µM	N-Acetylcysteine	1 mM
CTL	--	N/A	--	N/A

6b.5.1 Effect of Anti-Oxidant Feeding on Growth of Producer Cell Lines

Integral Viable Cell Densities (IVCD) of producer cell lines 7C3, 6A8 and APC at 96 h ("Early"), 168 h ("Mid"), and 288 h ("Late") of a fed-batch culture process are shown in Figure 6b.6, and it can be seen that there are commonalities in the cell line growth phenotypes when treated with some of the feeding strategies. By 96 h in culture, there were no significant impacts on the growth of 7C3 cells (Fig 6b.6.A) or of 6A8 cells (Fig.6b.6.B), and in APC only feeding strategies ζ and η had a slight negative impact on growth, with observed IVCD values of 44.9 cell•h/mL and 42.2 cell•h/mL, respectively, in comparison to the untreated cultures' IVCD of 60.0 cell•h/mL ($p<0.05$). Few of the feeding strategies had an impact on 7C3 cultures at any stage of growth. Indeed, only α is observed to have an impact on growth at early stationary phase, with IVCD at a lower value of 538 cell•h/mL compared to the untreated cultures IVCD value of 621 cell•h/mL ($p<0.05$). As the cells progressed

into late stationary phase, feeding strategies α and β elicited a positive impact on the growth of the cells. IVCD values for these cultures at 288 h were 999 cell•h/mL and 1014 cell•h/mL for α and β, respectively, compared to the IVCD of untreated cultures of 847 cell•h/mL ($p<0.01$).

In contrast to its sister cell line, cultures of 6A8 were affected in the middle and later stages of culture by all of the feeding strategies employed (Fig.6b.6.B). At 168 h into culture, cultures treated by all feeding strategies employed had lower IVCD values than the control culture of 532 cell•h/mL. Feeding strategies β and ε had the largest negative impact, with IVCD values of 405 cell•h/mL and 396 cell•h/mL, respectively ($p<0.01$), whilst γ, α, δ and ζ saw similar levels of reduction in IVCD (437 cell•h/mL, 443 cell•h/mL, 453 cell•h/mL and 451 cell•h/mL, respectively, $p<0.01$). The feeding strategy which had the least impact on early stationary phase of growth was η, with an observed IVCD value of 461 cell•h/mL ($p<0.05$). As the cells moved forward into late stationary phase, feeding strategies γ, δ, ε, ζ and η continued to elicit a reduction in IVCD compared to that of the untreated cultures' 779 cell•h/mL, with observed values of 687 cell•h/mL, 717 cell•h/mL, 649 cell•h/mL, 692 cell•h/mL and 705 cell•h/mL, respectively ($p<0.01$). However, cell cultures treated with feeding strategies α and β saw a large increase in growth between the 168 h and 288 h time points, with IVCD values at the latter recorded at 1019 cell•h/mL and 932 cell•h/mL, respectively ($p<0.01$). The response of 6A8 to feeding strategy α in terms of cell growth was thus very similar to that of 7C3 cultures.

As with 6A8, feeding strategies α and β led to a decrease in IVCD of APC cultures at 168 h (values of 383 cell•h/mL and 354 cell•h/mL, respectively, compared to untreated IVCD of 475 cell•h/mL, $p<0.01$), but when cultures were observed at 288 h the growth had improved to a point where IVCD values were significantly higher than that of the control, with observed values of 877 cell•h/mL and 852 cell•h/mL compared with 636 cell•h/mL in untreated cultures ($p<0.01$) (Fig.6b.6.C). Again as with 6A8, IVCD values of APC cultures treated with feeding strategies γ and ε were decreased at 168 h, standing at 413 cell•h/mL and 425 cell•h/mL, respectively ($p<0.01$).

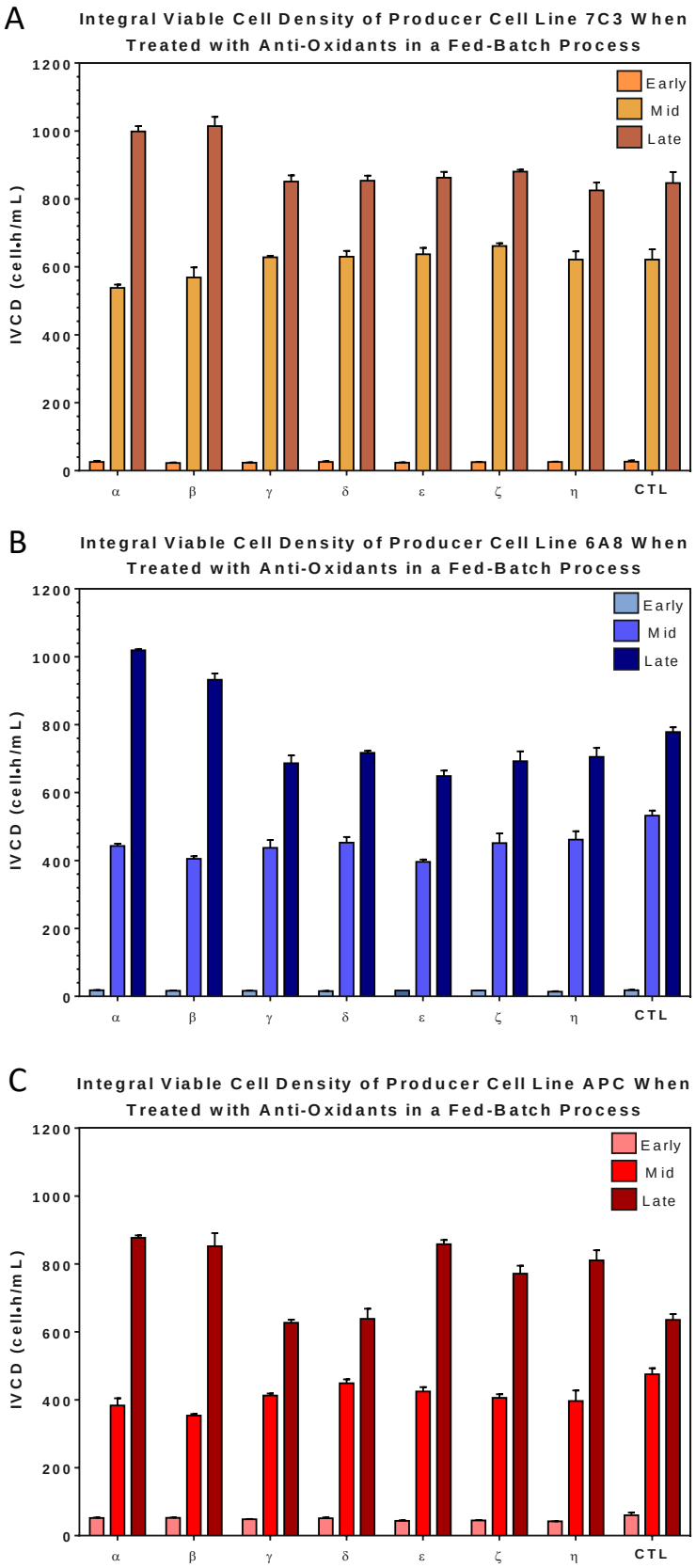

Figure 6b.6. (A) Integral Viable Cell Density at 96 h ("Early"), 168 h ("Mid") and 288 h ("Late") of 7C3 cultures treated with anti-oxidants in a fed-batch culture. (B) Integral Viable Cell Density at 96 h ("Early"), 168 h ("Mid"), and 288 h ("Late") of 6A8 cultures treated with anti-oxidants in a fed-batch culture. (C) Integral Viable Cell Density at 96 h ("Early"), 168 h ("Mid"), and 288 h ("Late") of APC cultures in a fed-batch process. Data bars indicate the Mean Average of triplicate cultures, with error bars indicating Standard Error of the Mean.

However, as the cells progressed to late stationary phase, cultures treated with γ no longer showed a difference to those untreated cultures, suggesting that growth had recovered somewhat, and in fact cultures treated with ε had recovered so much as to now show a higher level of growth at this later time point (IVCD value of 858 cell•h/mL, $p<0.01$). Despite showing retarded growth in the early stages of culture, at 288 h those cultures treated with feeding strategies ζ and η had overtaken the growth of untreated cells, with observed IVCD values of 771 cell•h/mL and 811 cell•h/mL, respectively.

6b.5.2 Effect of Anti-Oxidant Feeding on Productivity of Producer Cell Lines

When analysing the productivity of the producer cell lines treated with anti-oxidants in this second trial, it is clear that the improvements in growth seen when cultures were treated with feeding strategies α and β came at the expense of productivity. Figure 6b.7 and 6b.8 indicate the recombinant product titre and cell specific productivity of these cultures, respectively. [rP] and q_P are shown for 168 h ("Day7") and 240 h ("Day11") of culture for all three cell lines, and additional data is given for 6A8 cell cultures treated with α and β at 288 h (Day12), due to these cultures growing at a viability above 75 % for two days longer than any other culture.

In all three cell lines, treatment with feeding strategies α and β leads to a decrease in [rP] at 240 h. In 7C3 the reduction is proportionally the largest, with a drop in product titre at this time point from 257.1 μg/mL to 108.89 μg/mL and 123.79 μg/mL, respectively ($p<0.01$). The reduction seen in APC cultures treated with α and β is almost as drastic, with observed titres dropping from the 1836.81 μg/mL seen in untreated cultures to 1170.9 μg/mL and 1188.45 μg/mL, respectively ($p<0.01$). Reductions seen in 6A8 cultures treated with these feeding strategies are proportionally less than the other two cell lines, but still substantial; untreated 6A8 cultures at 240 h have a [rP] of 643.96 μg/mL, whereas the [rP] of α-treated cultures was 470.11 μg/mL, and of β-treated cultures was 473.15 μg/mL. However, unlike the other two cell lines, 6A8 cultures treated with α and β feeding strategies had extended culture periods, and so by the time the cells reached 288 h (12 Days), their [rP] had surpassed that of the untreated cultures, now being observed at a much higher 773.10 μg/mL and 706.32 μg/mL, respectively. When comparing these data to the cell specific productivity of cell lines treated with these feeding strategies, a clear decrease is seen at 240 h in 6A8 and APC cultures treated with α, with the former cultures seeing a drop from 0.827 pg/cell•h to 0.742 pg/cell•h ($p<0.01$) and the latter a drop from 2.59 pg/cell•h to 1.96 pg/cell•h ($p<0.05$) (Fig6b.8.B & C.). There is also a reduction of q_P at this time point in APC cultures treated with feeding strategy β, with a calculated q_P value of 2.049 pg/cell•h ($p<0.05$). There is no significant difference in the q_P of 7C3 cultures treated with either α or β in comparison to the untreated cultures (Fig.6b.8.A),

and indeed no significant difference between the q_P of 6A8 cultures at 240 h whether untreated, or treated with β.

The only feeding strategy that seems to produce a positive effect on [rP] in any of the cell lines is that of 7C3 cultures treated with δ. In this case, there is an observed increase at 240 h to 281.49 µg/mL ($p<0.1$), which correlates with an increase in qP at the same time point from 0.128 pg/cell•h to 0.292 pg/cell•h ($p<0.01$). In 7C3 ζ-treated and η-treated cultures, there was a decrease in [rP] at this time point to 242.89 µg/mL and 151.96 µg/mL, respectively ($p<0.01$), however this correlated with an increase in q_P in these cultures to 0.276 pg/cell•h and 0.185 pg/cell•h ($p<0.01$). Despite γ-treated and ε-treated 7C3 cultures having no observed difference in [rP] compared to untreated cultures, at 240 h there was an observed increase in q_P to 0.292 pg/cell•h and 0.284 pg/cell•h, respectively ($p<0.01$).

6A8 cultures treated with feeding strategies δ, ε and η saw a decrease in [rP] at 240 h, with observed titres of 572.2 µg/mL, 5538.19 µg/mL and 499.03 µg/mL, respectively ($p<0.01$), as did 6A8 ζ-treated cultures (598.8 µg/mL, $p<0.05$). The latter [rP] decrease correlated with a decrease in q_P, with values for ζ-treated cultures calculated at 0.710 pg/cell•h ($p<0.01$). Other than α and β, there were no feeding strategies that had a significant impact on APC [rP]. However, at 240 h, APC δ-treated cultures did see a drop in q_P, with a calculated specific productivity value of 2.23 pg/cell•h ($p<0.1$).

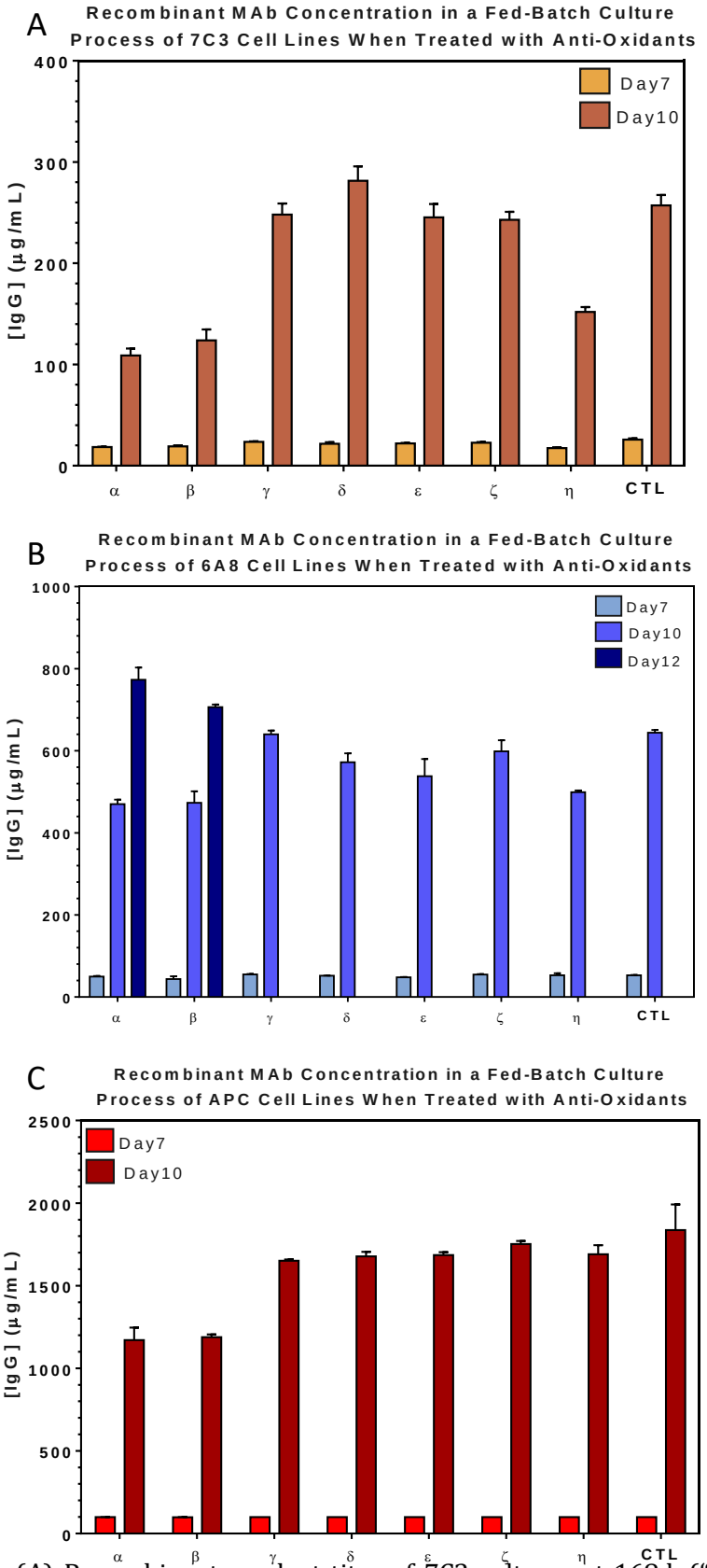

Figure 6b.7. (A) Recombinant product titre of 7C3 cultures at 168 h ("Day7") and 240 h ("Day10") in a fed-batch process when treated with anti-oxidants. (B) Recombinant product titre of 6A8 cultures at 168 h ("Day7"), 240 h ("Day10") and 288 h ("Day12") in a fed-batch process when treated with anti-oxidants. (C) Recombinant product titre of APC cultures at 168 h ("Day7") and 240 h ("Day10") in a fed-batch process when treated with anti-oxidants. Data bars show Mean Average of triplicate cultures, with error bars indicating Standard Error of the Mean.

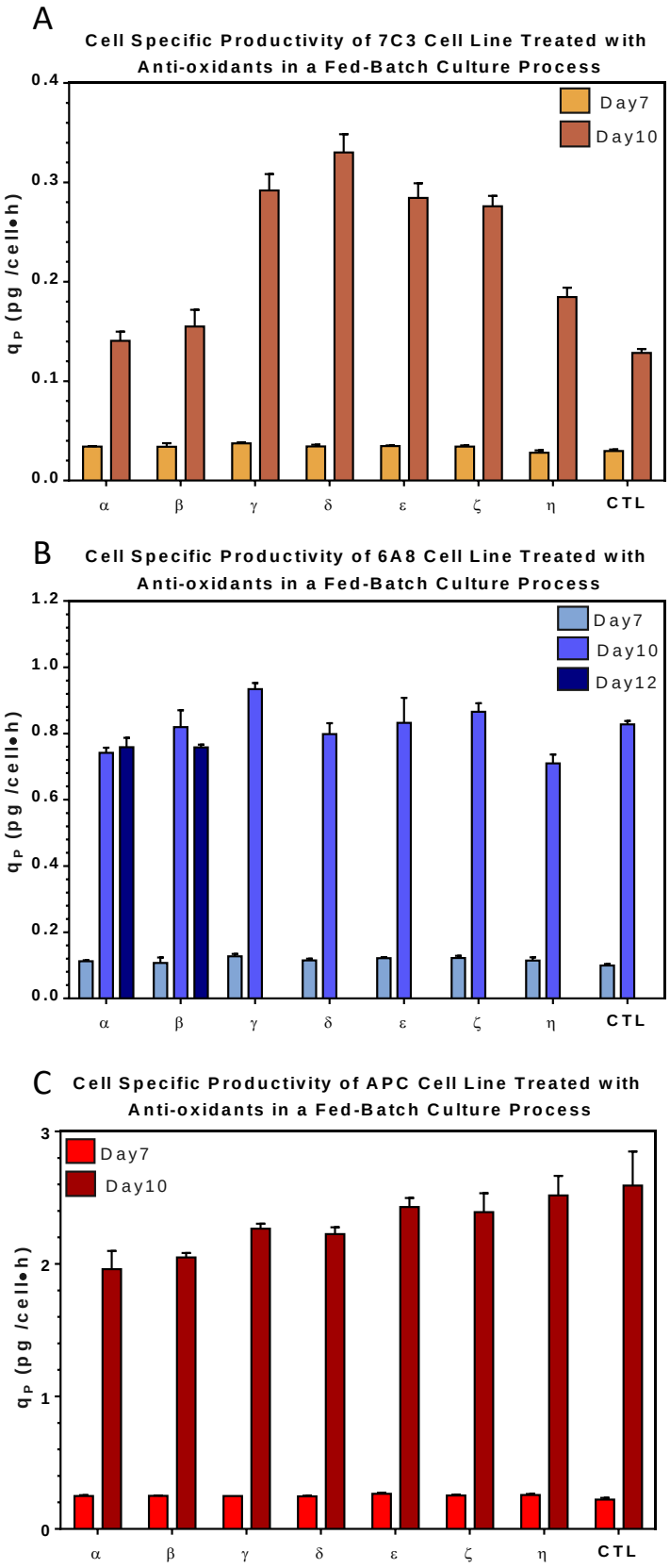

Figure 6b.8. (A) Cell Specific Productivity of 7C3 cultures at 168 h ("Day7") and 240 h ("Day10") in a fed-batch process when treated with anti-oxidants. (B) Cell Specific Productivity of 6A8 cultures at 168 h ("Day7"), 240 h ("Day10") and 288 h ("Day12") in a fed-batch process when treated with anti-oxidants. (C) Cell Specific Productivity of APC cultures at 168 h ("Day7") and 240 h ("Day10") in a fed-batch process when treated with anti-oxidants. Data bars show Mean Average of triplicate cultures, with error bars indicating Standard Error of the Mean.

6b.5.3 Effect of Anti-Oxidant Feeding on Reactive Oxygen Species Content of Producer Cell Lines

Figure 6b.9 shows the intracellular ROS levels of 7C3, 6A8 and APC cell lines at 144 h and 264 h as determined through CellROX staining and flow cytometry analysis. At the earlier stage in culture analysed, the only impacts on ROS levels are positive ones. When analysing 7C3 and 6A8 cultures at 144 h, treatment with both α and β feeding strategies elicited an increase in intracellular ROS levels. 7C3 cultures had observed fluorescence intensities of 5776 RFU when not treated, which then increased to 8873.5 RFU and 7298 RFU in α and β treated cultures, respectively ($p<0.01$) (Fig6b.9.A). In 6A8 cultures treated with these feeding strategies, fluorescence intensities were observed at 8235 RFU and 8051 RFU for α and β, respectively ($p<0.01$), compared with the intensity from untreated cultures of 2678 RFU (Fig.6b.9.B). However, as cultures moved to the latter stages of growth, ROS levels were decreased in comparison to the untreated cultures. In 7C3, α and β-treated cultures had fluorescence intensities of 2221 RFU and 2550 RFU at 264 h ($p<0.01$), down from 7374 RFU in untreated cultures. Untreated 6A8 cells at 264 h had observed fluorescence intensity of 6513 RFU, whereas α and β treated cultures had intensities of 3004 RFU and 2622 RFU, respectively ($p<0.01$). Similarly, APC cultures treated with feeding strategy β saw a reduction in ROS levels in late stationary phase in comparison to untreated cells, with an observed drop to 2145 RFU from 3676 RFU at 264 h into culture ($p<0.1$).

Cells earlier in culture that saw significant differences in ROS levels consistently had raised levels in comparison to their untreated cultures. In addition to α and β, feeding strategy δ elicited an increase in ROS levels at 144 h to 8496 RFU ($p<0.01$). Additionally, APC cells treated with feeding strategy ε saw an increase in ROS levels at 144h, with an observed increase from 2742 RFU when untreated to 5015 RFU ($p<0.1$). Conversely, significant alterations to ROS levels at later stages in culture were consistently negative. At 264 h, 6A8 cells treated with feeding strategy ε saw a decreased fluorescence intensity to 5053 RFU ($p<0.05$), while η-treated APC cultures saw decreased fluorescence intensity of 2104 RFU ($p<0.1$) at the same time point.

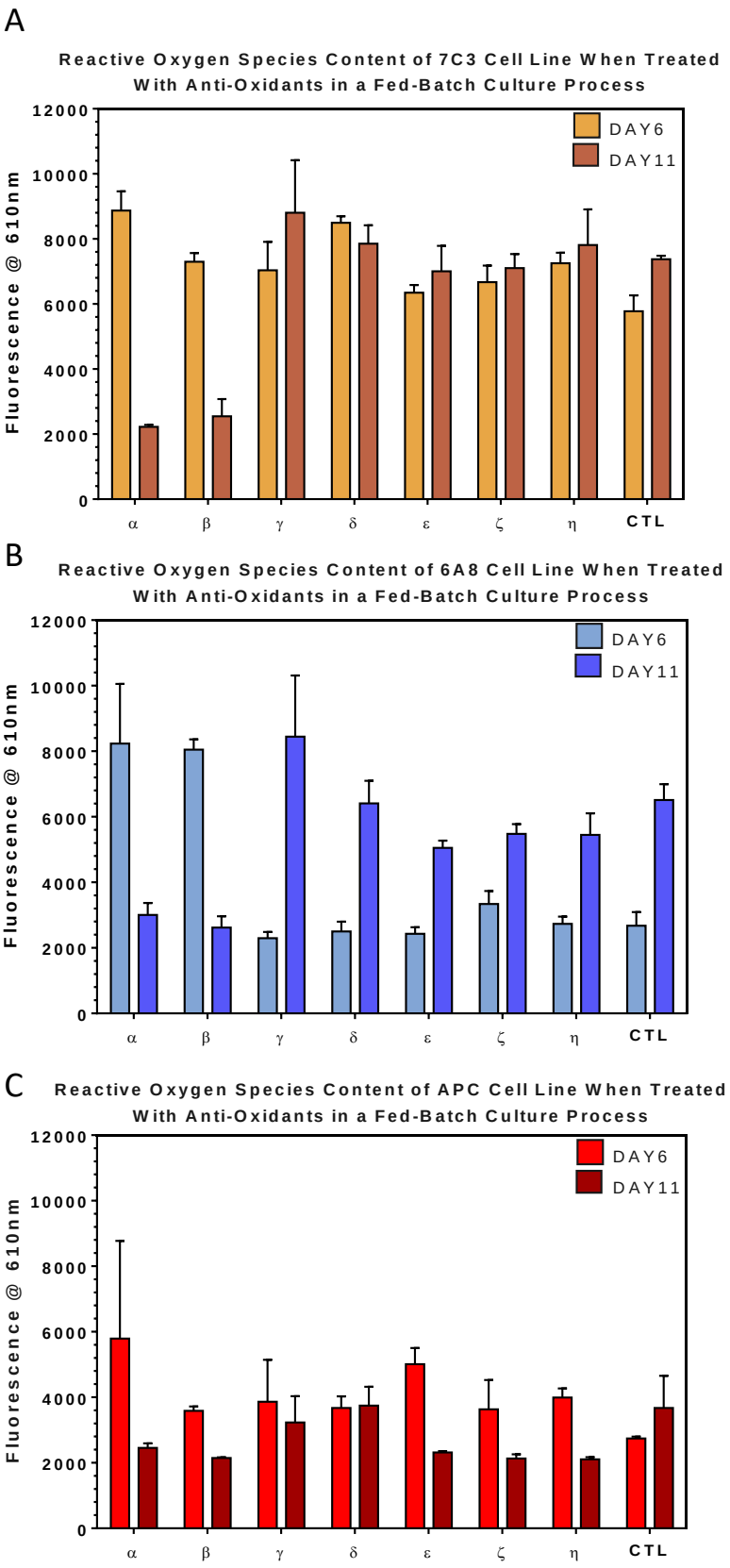

Figure 6b.9. (A) CellROX Fluorescence Intensity at 610 nm of 7C3 cultures at 144 h ("DAY6") and 264 h ("DAY11") in a fed-batch process when treated with anti-oxidants. (B) CellROX Fluorescence Intensity at 610 nm of 6A8 cultures at 144 h ("DAY6") and 264 h ("DAY11") in a fed-batch process when treated with anti-oxidants. (C) CellROX Fluorescence Intensity at 610 nm of APC cultures at 144 h ("DAY6") and 264 h ("DAY11") in a fed-batch process when treated with anti-oxidants. Data bars show Mean Average of triplicate cultures, with error bars indicating Standard Error of the Mean.

Chapter Summary

Data on producer cell lines 7C3 and 6A8's growth, productivity and intracellular ROS levels when treated with anti-oxidant chemicals in a batch-process were inputted into a DoE model in order to attempt to predict which anti-oxidant feeding strategies would be most likely to improve these characteristics in a fed-batch process. Cell line specific strategies were designed, however, some anti-oxidants dominated the feeding strategies of both cell lines due to common trends identified in the model. Since APC had not been screened for anti-oxidants in a batch process, a selection of the feeding strategies for both 7C3 and 6A8 were chosen to be screened in a fed-batch process of APC, meaning more anti-oxidant combinations were trialled in APC in this first screen than the other two cell lines.

Following the primary fed-batch anti-oxidant screen, cell lines were once again analysed for growth, productivity and intracellular ROS levels at various time points throughout culture. Trends of influence from some anti-oxidants during batch culture were further emphasised in fed-batch culture screens, and a move was made to attempt to engineer a single anti-oxidant feeding strategy that would positively impact the oxidative and productive characteristics of all three cell lines, rather than continuing with a cell line specific strategy. Cell lines were then again analysed for growth, productivity and intracellular ROS content. Feeding strategies containing L-Carnosine consistently had a positive impact on the growth and ROS levels of the producer cell lines trialled, however this appeared to be to the detriment of cellular productivity. Including α-lipoic acid in either the basal media or the feed also yielded some promising results, but not as consistently as those seen in L-Carnosine treatment. Perhaps most surprisingly, glutathione supplementation did not appear to have any significant impact on the growth, productivity, or oxidative metabolism of the producer cell lines.

Chapter 7:

Directed Evolution of Host CHO Cell Lines

Contents

List of Abbreviations

DE	Directed Evolution	**[rP]**	Recombinant Product Titre
CHO	Chinese Hamster Ovary	**q_P**	Cell Specific Productivity
H_2O_2	Hydrogen Peroxide	**ROS**	Reactive Oxygen Species
VCD	Viable Cell Density	**RFU**	Relative Fluorescence Units
IVCD/$IVCD_{MAX}$	Integral Viable Cell Density/Maximum	**BSO**	Buthionine Sulfoximine
ECL	Evolved Cell Line	**ME**	Menadione
C2A	Original Host Cell Line	**MTX**	Methotrexate
CTL	Co-culture Control Host Cell Line	**ECL_{SP}**	Producing Evolved Cell Line
AHC	Apollo Host Cell Line	**$C2A_{SP}$**	Producing Original Host Cell Line
MAb	Monoclonal Antibody		

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7.1 Introduction

The use of Directed Evolution (DE) as a tool for cell line development is one that is perhaps little used in industry, but in actuality is a simple and potentially powerful method of generating cell lines with specified characteristics without requiring a full mechanistic understanding of the origin of these characteristics. For example, recent work by K. Syddall in the D. C. James group (James, 2016) displayed cells that had evolved in a hypothermic environment to become larger and higher producers than their predecessors; it was not necessary to understand all of the mechanistic underpinnings of improved productivity in hypothermic environments, but higher performing cells were developed using an extremely simple methodology. While genetic engineering is a powerful, often used tool in cell line development (see Figueroa *et al,* 2001; Bailey *et al,* 1996; Fukuta *et al*, 2000 for examples), the 'omics era has introduced the idea that altering specific biochemical pathways may not be the most effective strategy, and instead the focus should be on global activities such as redox balancing and cellular regulatory elements (Tyo *et* al, 2007). With respect to targeting of oxidative defence mechanisms through DE approaches, Spitz *et al* (1988) evolved Chinese Hamster Fibroblast cells with higher catalase levels than its parental cell line through chronic exposure to Hydrogen Peroxide, without requiring genetic or e pigenetic understanding of the transcription of catalase genes and the pathways that lead to their transcription.

Chinese Hamster Ovary (CHO) Host cell line C2A underwent chronic exposure to Hydrogen Peroxide (H_2O_2) over a period of roughly 130 generations in an attempt to encourage evolution towards a more oxidative stress resistant phenotype. H_2O_2, as a ROS molecule in itself, thus provided an evolutionary pressure to adapt to higher base line levels of ROS and thus a higher oxidative stress level, therefore allowing for selection of cells with improved oxidative defence mechanisms. These improvements could present in a reduction of intracellular ROS production to reduce the base line ROS levels, or in cells with an increased production of anti-oxidant enzymes such as catalase or superoxide dismutase. Since host cells were used, there were no measurable productivity characteristics during the evolution process, and as such cells were selected based on their improved growth characteristics. Cells were transiently transfected with circular DNA to analyse growth and productivity characteristics post-evolution in a high-throughput manner. Stable transfectant pools were also generated for a more in-depth analysis of growth and productivity characteristics in a fed-batch process conducted.

7.2 Experimentation

Host cell line C2A was revived from cryopreservation and maintained at a 30mL volume in shaking flask culture according to Chapter 3 "*Materials and Methods*", Section 3.2.2 "*Maintenance and*

Routine Subculture", pg34. Cells were evolved through chronic exposure to H_2O_2 according to Section 3.5.1 "*Evolution Strategy*", pg44. Transfection protocol optimisation was conducted according to Section 3.5.3 "*Transient Transfection Optimisation*", pg46. Transient transfection through electroporation was conducted and cells subsequently analysed for growth, productivity and ROS levels according to 3.5.4 "*Transient Transfection*", pg48. Stable pools were generated according to Section 3.5.5 "*Stable Transfection*", pg49, and subsequent transfected cell lines cultured in a fed-batch process according to Section 3.2.2.2 "*Fed-batch Feeding Strategy: FUJIFILM Diosynth Biotechnologies*", p36. Cells were fixed and stained for flow cytometry analysis according to Section 3.4.3 "Reactive Oxygen Species Quantification", pg43, and analysed using a FASCAria II Flow Cytometer according to Sub-Section 3.4.3.2 "Imaging Using Flow Cytometry", pg44.

7.3 Directed Evolution Methodology

Figure 7.1 shows the Viable Cell Density (VCD), Percentage Viability and growth rate of host cells lines both undergoing evolution pressure over time and control cells being grown for the same time but without evolution pressure. Initially, Hydrogen Peroxide (H_2O_2) was introduced to cultures at the IC_{50} of 250 µM, with fresh H_2O_2 being introduced at each subculture (passage) every 3 days. Once cells were deemed to have recovered (VCD not significantly different to control culture, or Percentage Viability above 80 %), H_2O_2 concentration was doubled. Thus, the concentration of H_2O_2 being introduced to cultures was increased to 500 µM at Generation N^{o} 13, to 1 mM at Generation N^{o} 66 and to 2 mM at Generation N^{o} 96. Cells struggled to recover when H_2O_2 was increased to 2 mM, and so H_2O_2 was removed from media at Generation N^{o} 98 to allow cells to recover, before reintroducing H_2O_2 at the same concentration of 2 mM at Generation N^{o} 108. At Generation N^{o} 130, H_2O_2 was removed from cultures, and cells banked.

7.4 Transient Transfection of Evolved Cell Line

7.4.1 Transfection Optimisation

In order to select the appropriate 4D-Nucelofector Protocol, cell number and DNA concentration for transfection, optimisation was conducted according to the instructions provided for the 4D-Nucleofector from Lonza (See Chapter 3 "*Materials and Methods*", Section 3.5.3, "*Transient Transfection Optimisation*", pg46). Supernatent samples were analysed using the Octet for IgG binding rate (an indirect indicator of relative productivity), and the results are indicated in Figure 7.2. The protocol and cell/DNA combination that indicated the highest productivity was EH-100, with 9.6 x10^6 cells/mL and 15 µg DNA per transfection (circular or linearised, depending on the transfection being conducted).

7.4.2 Growth and Productivity Characteristics of Transiently Producing Evolved Cell Lines

Evolved Cell Line (ECL), original host cell line (C2A), control host cell line (CTL) and Apollo Host Cell line (AHC) were transfected with circular DNA containing a recombinant MAb product. All cell lines were then transferred to 6 well plates and cultured for 6 days, with samples taken to measure cell density, viability, and productivity taken on Day4 (96h post transfection) and Day6 (148h post transfection). Cell samples were also stained and fixed for flow cytometry analysis and Reactive Oxygen Species levels on Day3 (72h post transfection) and Day6 (148h post transfection).

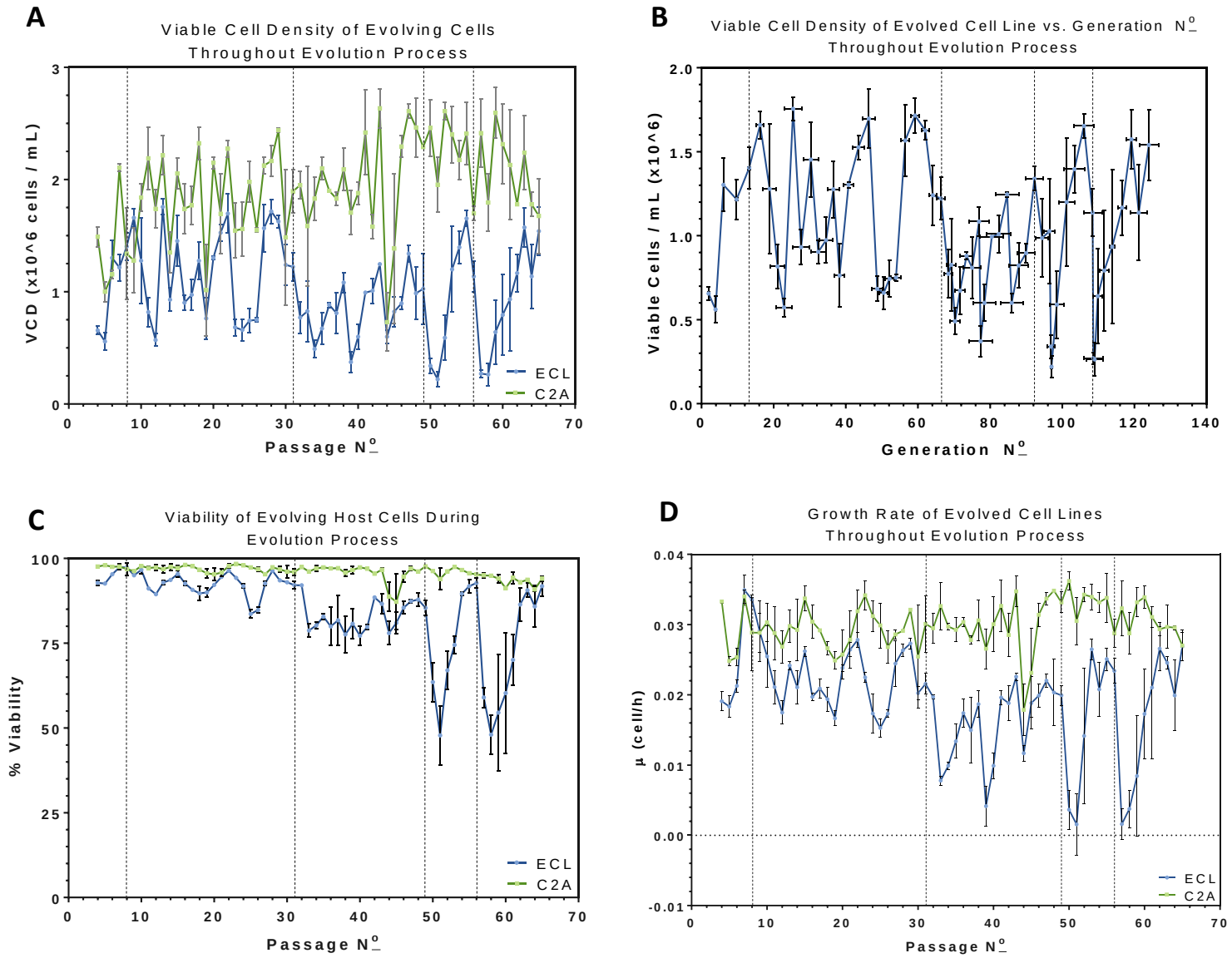

Figure 7.1. (A) The Viable Cell Density of the Evolved Cell Line (ECL) and the original Host Cell Line (C2A) up to Passage No 65. (B) Viable Cell Density of Evolved Cell Line up to Generation No 130. (C) % Viability of the Evolved Cell Line (ECL) and original Host Cell Line (C2A) up for Passage No 65. (D) Growth rate (μ) of Evolved Cell Line (ECL) and original Host Cell Line (C2A) up to Passage No 65. Dashed lines indicate introduction of H_2O_2 at an increased concentration. Data Points are Mean average of triplicate cultures, with error bars indicating Standard Error of the Mean.

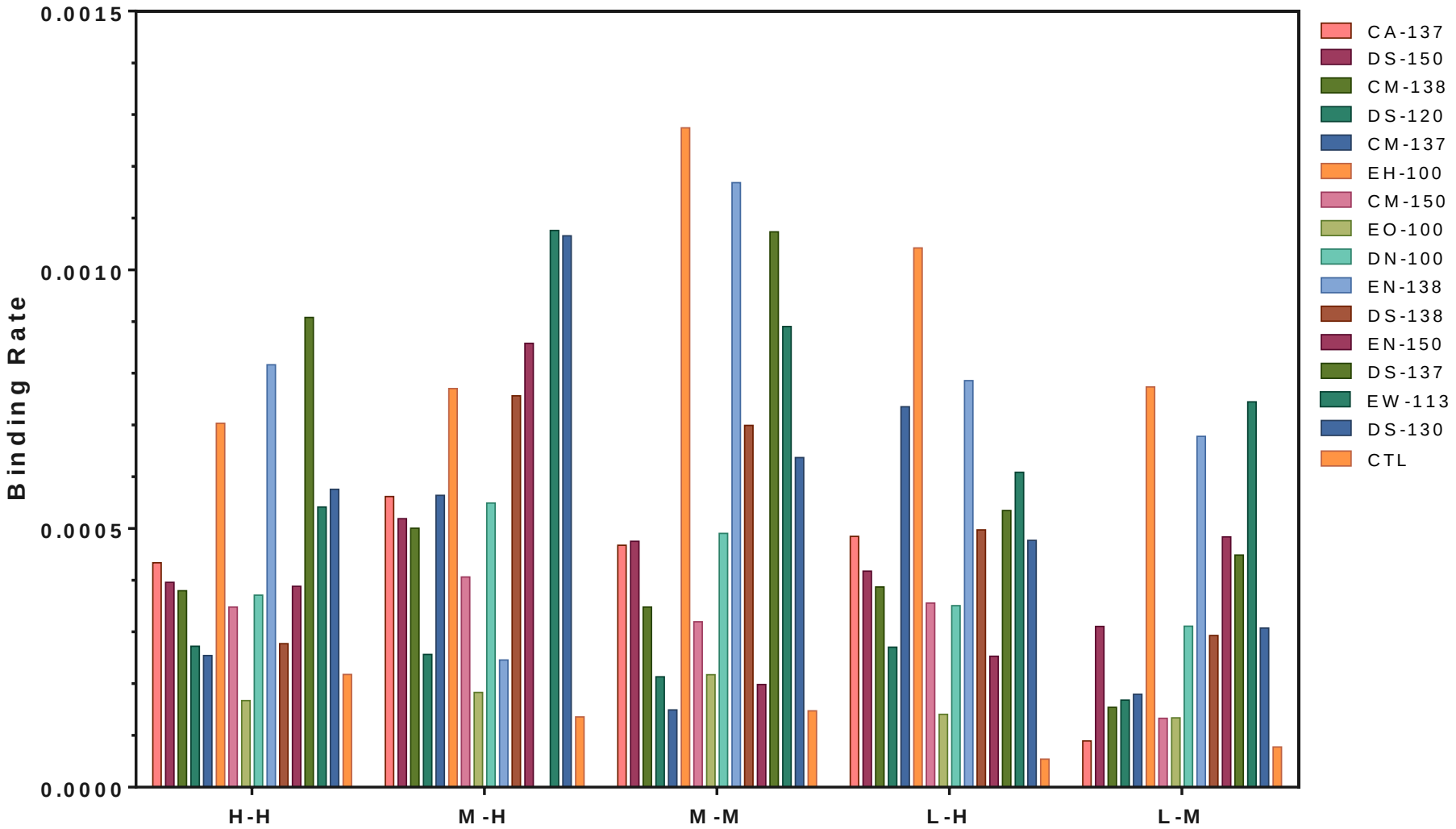

Figure 7.2. Transfection protocol optimisation. Combinations of High (16 x10^6 cells/mL), Medium (9.6 x10^6 cells/mL) and Low (3.2 x10^6 cells/mL) concentrations of cells with High (22 µg) and Medium (15 µg) quantities of circular DNA were trialled in the 15 different electroporation protocols in the 4D-Nucleofector, with one control well transfected without the presence of DNA. Supernatant samples were analysed for their IgG binding rate using an Octet.

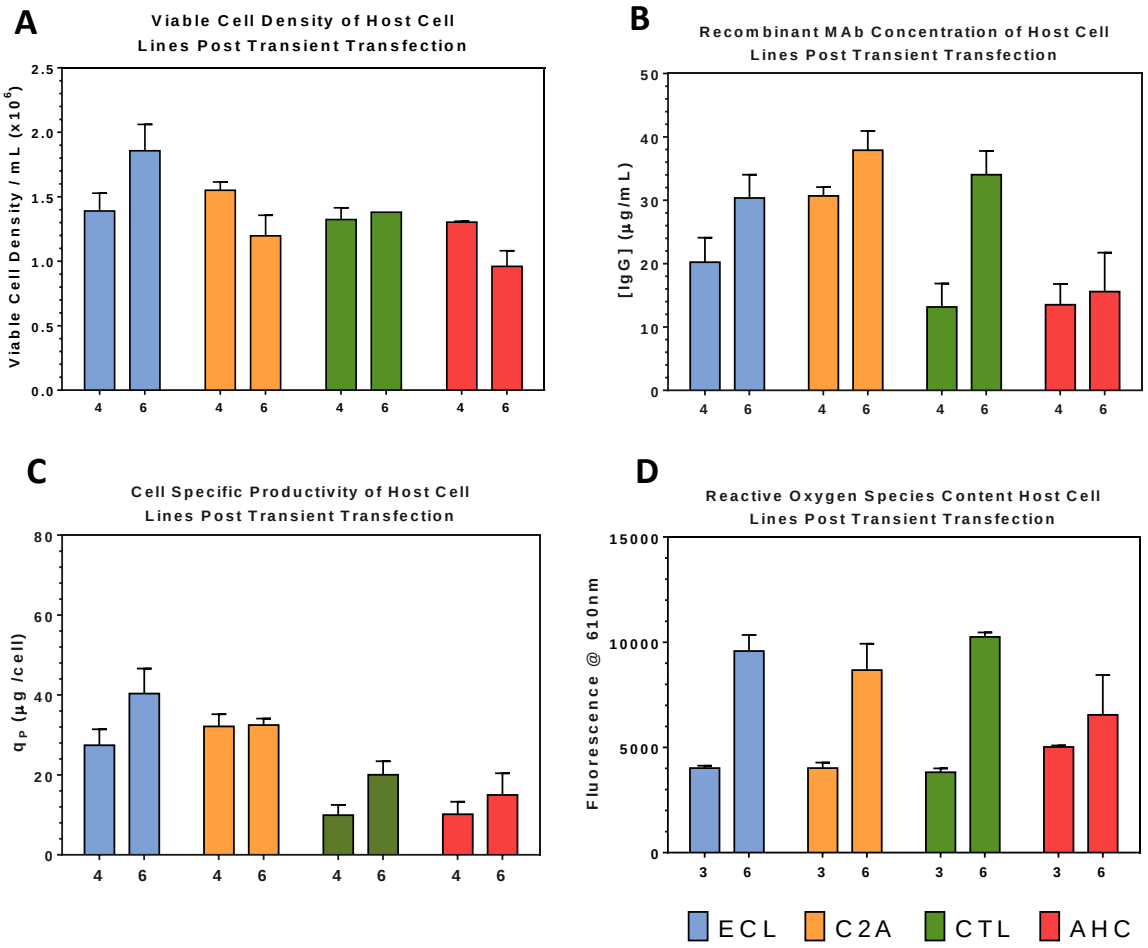

Figure 7.3. (A) Viable Cell Density of host cell lines ECL, C2A, CTL, AHC at 96 h (4) and 148 h (6) post transfection. (B) rProduct titre of host cell lines ECL, C2A, CTL, AHC at 96 h (4) and 148 h (6) post transfection. (C) Cell specific productivity of host cell lines ECL, C2A, CTL, AHC at 96 h (4) and 148 h (6) post transfection. (D) Fluorescence intensity of CellROX stained cell samples from host cell lines ECL, C2A, CTL, AHC at 72 h (3) and 148 h (6) post transfection. Data bars indicate Mean average of triplicate cultures, and error bars indicate Standard Error of the Mean.

For the majority of host cell lines post transient transfection, cell growth in 6 well plates on Day6 will be either lower than or similar to that measured earlier at Day3 of culture. However, the evolved cell line (ECL) increased its VCD 1.3-fold between Days 3 and 6. VCD for the evolved cell line at Day6 was also significantly higher than the other three cell lines, with observed VCD values of 1.2 x10^6 cells/mL ($p<0.05$), 1.38 x10^6 cells/mL ($p<0.05$) and 0.96 x10^6 cells/mL ($p<0.01$) for cell lines C2A, CTL and AHC, respectively. This data shows improved growth during transient expression for the evolved cell line, even though the protocol was optimised for the original host cell line. There was no significant difference in the growth characteristics of the original host cell line with the control cell

line, indicating that the improvements seen in the ECL were due to the evolution pressure applied, and not simply because of random genetic drift during the extended passaging period.

In terms of productivity, there were smaller differences observed between the transiently transfected host cell lines than in terms of growth. At 96 h post transfection, there was a lower product titre ([rP]) observed in the ECL cultures than that of the C2A cultures, with [rP] measured at 20.22 µg/mL compared to 30.70 µg/mL ($p<0.05$), however despite a slight difference at 148 h of 30.35 µg/mL compared to 37.91 µg/mL, this difference was no longer statistically significant (Fig.7.3.B). Product titre for ECL was also higher at 148 h than AHC, with the observed [rP] for AHC measured at 15.59 µg/mL ($p<0.05$). There was no significant difference observed between the cell specific productivity (q_P) of ECL and of C2A. When compared to CTL and AHC, however, ECL had a much higher q_P throughout the post transfection culture period, measuring at 27.43 pg/cell at 96 h and 40.35 pg/cell at 148 h, compared with 9.94 pg/cell and 20.06 pg/cell in CTL cultures and 10.17 pg/cell and 15.0 pg/cell in AHC cultures ($p<0.01$). In terms of product titre, C2A had a significantly higher titre than CTL at 96 h but by Day6 there was no longer a significant difference between the two. The difference in growth profiles, however, meant that the calculated q_P for C2A was far higher than that of CTL at both time points, with final day q_P for C2A calculated at 32.46 pg/cell compared with 20.06 pg/cell for CTL ($p<0.01$) (Fig 7.3.C).

CellROX staining indicated an increase in ROS levels from 72 h post transfection to 148 h across all four cell lines, but there was very little difference between them (Fig 7.3.D). At 72 h (Day3), all cell lines had roughly equitable fluorescence intensities, measuring at 4021 RFU, 4022 RFU, 3822 RFU and 5019 RFU for ECL, C2A, CTL and AHC, respectively; AHC had slightly elevated levels of ROS, but this was not significant. By 148 h (Day6), the fluorescence intensities for ECL and CTL were still roughly equitable (9582 RFU and 10255 RFU, respectively). C2A had a lower mean fluorescence reading of 5684 RFU, however due to sample variation there was no determined significance. There were indications of lower levels of ROS in AHC by the end of the post transfection culture period, however, with CellROX fluorescence intensity of 6552 RFU, which was significantly lower than ECL ($p<0.1$) and CTL ($p<0.05$).

7.4.3 Probing of Oxidative Metabolism of Evolved Cell Lines using Pro-Oxidant Chemicals

After transient transfection with circular DNA containing a recombinant MAb product, pro-oxidant chemicals were added to the culture wells in order to establish which of the four host cell lines trialled had the highest resistance to oxidative stress. Pro-oxidants Buthionine Sulfoximine (BSO), Menadione (ME) and H_2O_2 were used to probe the oxidative metabolism of the cell lines. To allow

the cells to recover from the electroporation process, pro-oxidants were added 24 h post transfection.

Figure 7.4 shows the [rP] and q_P of untreated ECL, C2A, CTL and AHC cell lines as well as the cell lines treated with pro-oxidant chemicals. There was no significant difference observed between the [rP] of the four cell lines when treated with 500 µM BSO (Fig.7.4.B). However, due to the large differences seen in growth profiles of cells treated with this pro-oxidant, there is an observed difference in cell specific productivity, with ECL having a significantly higher q_P than the other three cell lines at 96 h post transfection; on Day4, calculated q_P for ECL was 55.29 pg/cell, compared to 28.24 pg/cell for C2A ($p<0.1$), 15.66 pg/cell for CTL ($p<0.01$) and 20.53 pg/cell for AHC ($p<0.05$). CTL cultures treated with BSO had a consistent, but comparatively low q_P, with a largely unchanged specific productivity of 15.07 pg/cell at 148 h post transfection (compared to the 41.07 pg/cell seen in the BSO-treated ECL cultures, $p<0.05$). At 96 h post transfection, ME-treated ECL cultures showed a significantly higher product titre than AHC cultures: 24.74 µg/mL compared to 9.3 µg/mL. ($p<0.1$) However, by Day6, CTL cultures showed the highest titre, at 36.26 µg/mL compared to the 15.71 µg/mL of ME-treated ECL ($p0<0.05$). C2A, cultures, too, had significantly higher titres than ECL at this time point, with a measured [rP] of 27.63 µg/mL ($p<0.1$). Once again, q_P for ECL cultures at 96 h post transfection (54.84 pg/cell) was higher in this treated batch than the others, CTL (17.52 pg/cell, $p<0.05$) and AHC (6.71 pg/cell, $p<0.05$) cultures had much lower observed q_P at this time point. However, by the time cultures reached Day6, there was no longer an observable significant difference in the q_P of any of the cell lines trialled.

Interestingly, ECL cultures appeared to perform better or equally well compared to other cell lines when treated with BSO and ME, chemicals not used in the directed evolution process. However, when treated 24 h post transfection with 250µM H_2O_2, ECL cultures saw a slightly diminished productivity in comparison to some of the other cell lines. In terms of product titre, C2A had the highest titre 96 h post transfection, with an observed [rP] of 33.96 µg/mL. This was significantly higher than that of ECL cultures at the same time point, with an observed [rP] in these cultures of 20.18 µg/mL ($p<0.1$). ECL cultures also showed a lower titre at 148 h post transfection, being significantly lower than the titre of CTL cultures (13.91 µg/mL compared to 27.96 µg/mL, $p<0.05$)). When calculating q_P, it can be seen that by Day6, there was no significant difference between the q_P of ECL cultures (23.81 pg/cell) and that of any of the other cell lines. Earlier in culture, however, cell specific productivity was significantly reduced in comparison to the original host, with a calculated q_P for H_2O_2-treated ECL at 96h of 37.55 pg/cell compared with 50.95 pg/cell for C2A ($p<0.1$).

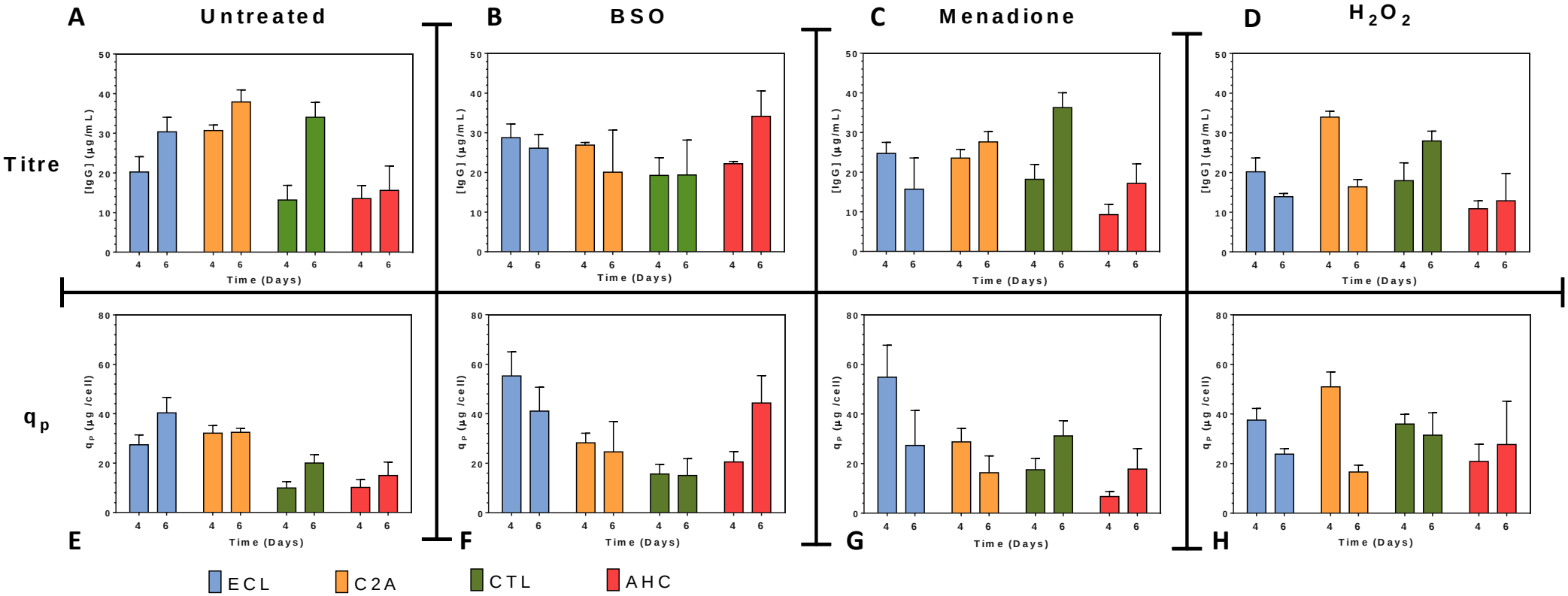

Figure 7.4. Host Cell Lines ECL, C2A, CTL and AHC treated with pro-oxidant chemicals 24 h post transfection with circular DNA. (A) Product titre on Day4 and Day6 of culture without treatment. (B). Product titre on Day4 and Day6 of culture when treated with BSO. (C) Product titre on Day4 and Day6 of culture when treated with ME. (D). Product titre on Day4 and Day6 of culture treated with H_2O_2. (E). Cell Specific Productivity on Day4 and Day6 of culture without treatment. (F). Cell Specific Productivity on Day4 and Day6 of culture treated with BSO. (G). Cell Specific Productivity on Day4 and Day6 of culture when treated with ME. (H). Cell Specific Productivity on Day4 and Day6 of culture when treated with H_2O_2. Data bars indicate Mean average of triplicate wells, with error bars indicating Standard Error of the Mean.

7.5 Generation of Stable Pools

7.5.1 Transfection Methodology

Cells from ECL and C2A cultures were transfected using the 4D-Nucleofector protocol identified during optimisation with a linearised version of the same plasmid used in previous transient experiments. Of the four transfections conducted, one of the C2A flasks was not successful at integrating the plasmid into nuclear DNA, the cells being unable to grow in the presence of selection pressure chemical Methotrexate (MTX). Post recovery, cells were transferred to shaking flask cultures, and this process cause one of the ECL cultures to become contaminated. Thus, all work was conducted on the remaining three cultures per cell line, ensuring statistical analysis was still possible. After transferral to shaking flask cultures, transfected cells were banked.

7.5.2. Fed-Batch Culture Characteristics of Cells Isolated From Stable Pools

7.5.2.1 Growth and Productivity

Cells isolated from the ECL stable pools (ECL_{SP}) and the C2A stable pools ($C2A_{SP}$) were cultured a fed-batch process in order to assess their relative growth and productivity characteristics post transfection. Figure 7.5 shows the IVCD, [rP] and q_P of ECL_{SP} and $C2A_{SP}$ cultures in a fed batch process at 72 h ("Early"), 168 h ("Mid") and 264 h ("Late") of culture. There is a clear improvement seen in the evolved cell cultures in terms of growth, since at all three time points indicated, IVCD is higher for ECL_{SP} than for $C2A_{SP}$ (Fig.7.5.A). At 72 h, observed IVCD for ECL_{SP} is 73.3 cell•h/mL compared with 64.2 cell•h/mL for $C2A_{SP}$ cultures. Although this result is not significant, the trend continues into later culture, with IVCD for ECL_{SP} and $C2A_{SP}$ at 168 h calculated at 659 cell•h/mL and 517 cell•h/mL, respectively ($p<0.05$), and at 264 h, 2101 cell•h/mL and 1766, cell•h/mL respectively ($p<0.01$).

There is no significant difference between the two cell lines when observing product titre at 72 h, 168 h and 240 h of culture, although there is a suggested trend of the $C2A_{SP}$ cultures having a higher titre than ECL_{SP}. The measured [rP] for ECL_{SP} and $C2A_{SP}$ at 72 h was 95.32 µg/mL and 100.30 µg/mL, respectively; at 168 h titre was 134.61 µg/mL and 138.02 µg/mL for the two cell lines, and at 264 h the observed [rP] were 153.41 µg/mL and 177.29 µg/mL. This similarity in product titre means that calculated cell specific productivity in ECL_{SP} cultures was lower than in $C2A_{SP}$, although only early on in culture was this difference significant. On Day3, q_P for $C2A_{SP}$ was 0.495 pg/cell•h compared to that of ECL_{SP} of 0.368 pg/cell•h ($p<0.05$). This trend continued, with calculated q_P for $C2A_{SP}$ and ECL_{SP} at 168 h of 0.268 pg/cell•h and 0.204 pg/cell•h, and at 264 h of 0.100 pg/cell•h and 0.073 pg/cell•h, respectively.

7.5.2.2. Reactive Oxygen Species Content

Cell samples were taken from fed-batch cultures of ECL_{SP} and $C2A_{SP}$ at 144 h (Day6) and 264 h (Day11) of a fed-batch culture to be fixed and stained with CellROX™ Deep Red Reagent, for Reactive Oxygen Species (ROS) level determination. In both cases, relative CellROX™ fluorescence intensity was lower for ECL_{SP} cultures than it was for $C2A_{SP}$, indicating a lower level of intracellular ROS in the evolved cell line (Fig.7.5.D). At 144 h, fluorescence intensity was at 4712 RFU in ECL_{SP} cultures, compared with 10467 RFU in $C2A_{SP}$ cultures ($p<0.05$), and at 264 h fluorescence intensity for ECL_{SP} and $C2A_{SP}$ was 21674 RFU and 30790 RFU, respectively ($p<0.1$).

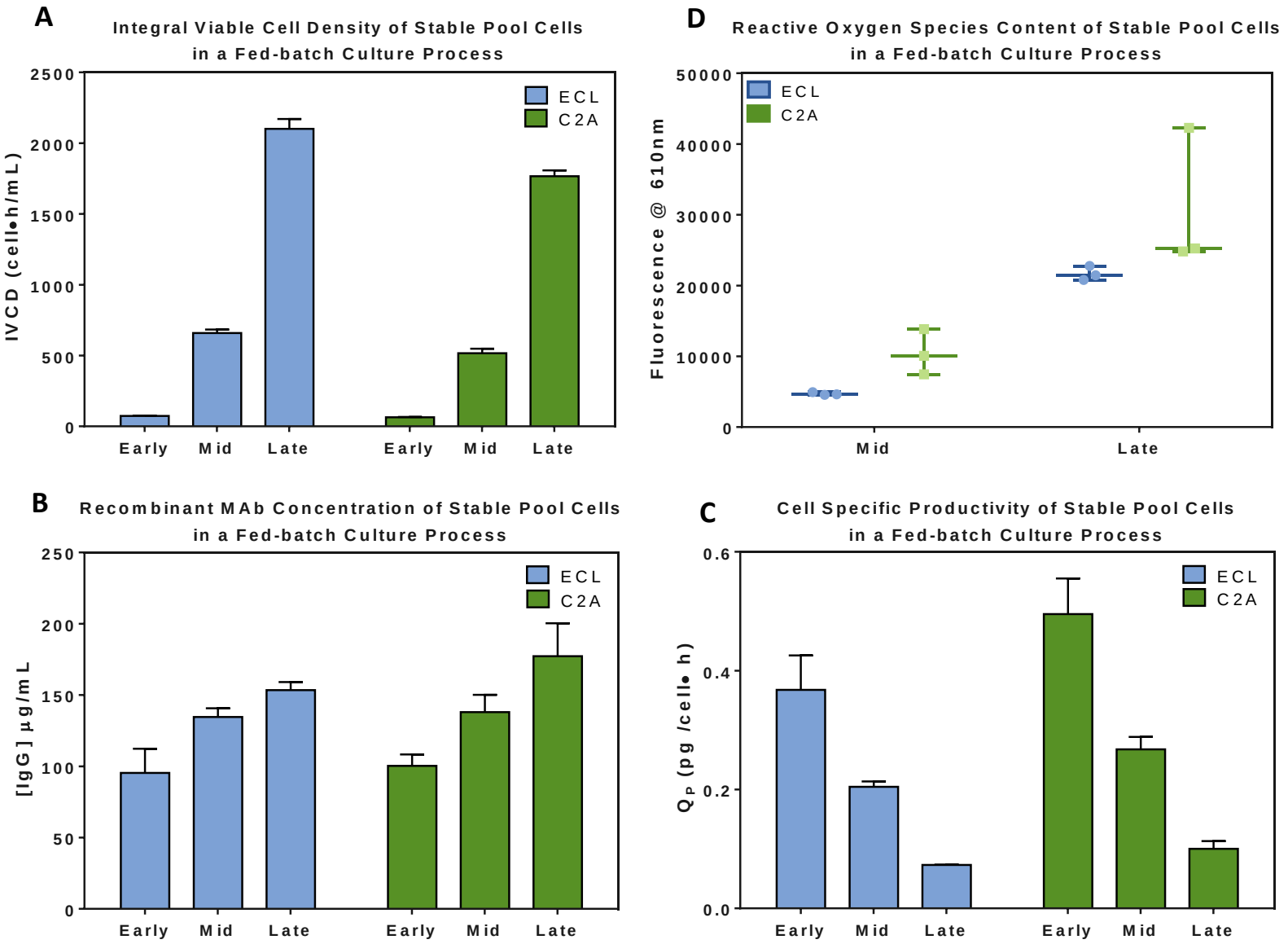

Figure 7.5. (A) Integral Viable Density of ECL_{SP} and $C2A_{SP}$ in a Fed-batch culture process at 72 h ("Early"), 168 h ("Mid") and 264 h ("Late") of culture. (B). Recombinant product titre of ECL_{SP} and $C2A_{SP}$ in a Fed-batch culture process at 72 h ("Early"), 168 h ("Mid") and 264 h ("Late") of culture. (C). Cell Specific Productivity of ECL_{SP} and $C2A_{SP}$ in a Fed-batch culture process at 72 h ("Early"), 168 h ("Mid") and 264 h ("Late") of culture. . (D). Reactive Oxygen Species content of ECL_{SP} and $C2A_{SP}$ in a Fed-batch culture process at 168 h ("Mid") and 264 h ("Late") of culture.

7.5.3 Probing of Oxidative Metabolism of Evolved Cell Lines using Pro-Oxidant Chemicals

ECL_{SP} and $C2A_{SP}$ cell lines were treated with pro-oxidant chemicals BSO, ME and H_2O_2 on Day0 of a batch culture process. Figure 7.6 shows the $IVCD_{MAX}$, final day recombinant product titre and cell specific productivity of the two cell lines after treatment compared with untreated cultures. There is a clear advantage seen in terms of growth when observing the $IVCD_{MAX}$ of the ECL_{SP} cell line in comparison with $C2A_{SP}$ (Fig.7.6.A). In untreated cultures, calculated $IVCD_{MAX}$ for ECL_{SP} was 421 cell•h/mL compared with 333 cell•h/mL seen in $C2A_{SP}$ ($p<0.1$). All the treated cultures also saw a higher level of growth in the ECL_{SP} cultures, with $IVCD_{MAX}$ for BSO, ME and H_2O_2 treated ECL_{SP} calculated at 54.2 cell•h/mL, 327 cell•h/mL and 142 cell•h/mL, respectively, compared to that of $C2A_{SP}$, which were observed at 49 cell•h/mL, 267 cell•h/mL and 74 cell•h/mL, respectively. While none of these results were statistically significant, it indicates a clear trend of increased capacity for growth under higher levels of oxidative stress in the ECL_{SP} cultures.

Productivity in ECL_{SP} cultures was also higher, with an observed [rP] of 44.7 µg/mL and q_P of 0.831 pg/cell•h, compared with that of 26.90 µg/mL ($p<0.1$) and 0.565 pg/cell•h ($p<0.01$) observed in $C2A_{SP}$ cultures. Observed [rP] for untreated cultures and ME-treated cultures, however, were very similar between the two cell lines; untreated ECL_{SP} and $C2A_{SP}$ [rP] was 47.49 µg/mL and 45.01 µg/mL, respectively, and in ME-treated cultures [rP] was 48.90 µg/mL and 50.76 µg/mL (Fig.7.6.B). There was a decrease in final day titre in both cell lines when treated with H_2O_2, although the decrease was less pronounced in ECL_{SP} than in $C2A_{SP}$, with observed [rP] in these cultures of 30.22 µg/mL and 14.32 µg/mL, respectively, although there was no calculated significant difference between the two cell lines. There was a similar pattern observed in the cell specific productivity of the untreated, ME-treated and H_2O_2-treated cultures. Calculated q_P was 0.119 pg/cell•h and 0.143 pg/cell•h for untreated ECL_{SP} and $C2A_{SP}$ cultures, respectively, and 0.152 pg/cell•h and 0.191 pg/cell•h in ME-treated cultures (Fig7.6.C). In H_2O_2 cultures, the calculated q_P was higher in the ECL_{SP} cell line (0.235 pg/cell•h) compared to $C2A_{SP}$ (0.162 pg/cell•h), although this again was not significant.

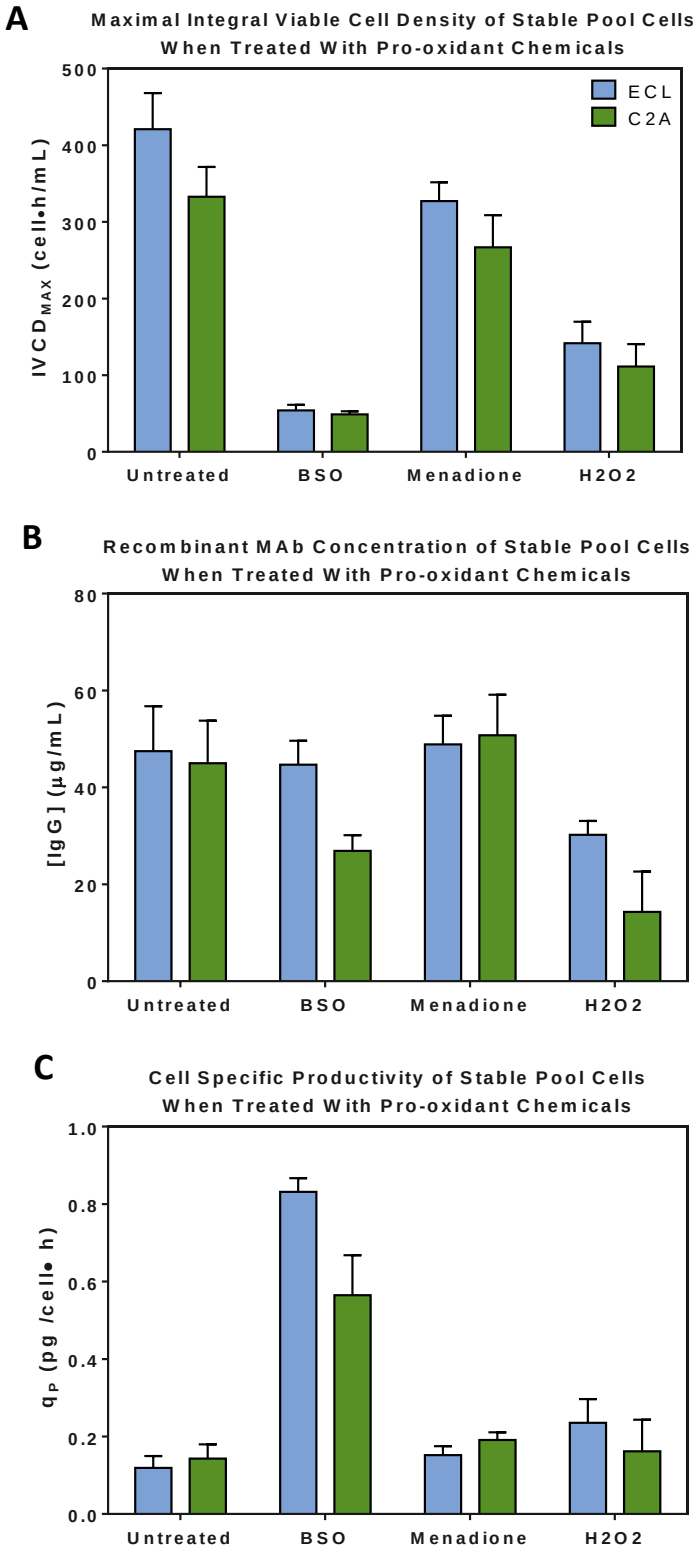

Figure 7.6. ECL_{SP} and $C2A_{SP}$ cell lines were grown in a batch culture process and treated with Buthionine Sulfoximine (BSO), Menadione and Hydrogen Peroxide (H_2O_2). (A) Maximum Integral Viable Cell Density, cell•h/mL. (B) Final Day Product Titre, µg/mL. (C) Cell Specific Productivity, pg/cell•h. Data bars indicate Mean average of triplicate flasks, with error bars indicating Standard Error of the Mean.

Chapter Summary

Host cell line C2A underwent chronic exposure to Hydrogen Peroxide (H_2O_2), a broad acting pro-oxidant chemical, in an attempt to direct cell line evolution towards increased oxidative stress resistance. The IC_{50} of H_2O_2 in C2A was established at 250 µM, but by the end of the evolution period, cells were growing at around 75 % viability in 2 mM H_2O_2, and above 80 % viability in 1.6 mM H_2O_2, indicating an improved capacity for growth under increased oxidative stress conditions. Triplicate flasks of C2A were co-cultured for the same number of passages as the evolved cell line, but without addition of the evolution pressure, in order to provide a control against beneficial changes to oxidative metabolism being due to random genetic drift.

Newly evolved host cell line (ECL) was transiently transfected with circular DNA containing a recombinant MAb product, as were the original host cell line (C2A), the co-cultured control cell line (CTL) and the Apollo Host Cell line (AHC). Product titre was largely the same for ECL, C2A and CTL in the 6 day culture period post transfection, but observed growth was higher in that of the ECL cultures. There was also very little difference in the Reactive Oxygen Species (ROS) content of these cell lines, while AHC appeared to have a lower ROS content overall. When treated with various pro-oxidant chemicals, any advantage ECL had over the other three cell lines was largely seen early on in culture, with [rP] increased on Day4 in Menadione (ME) treated ECL cultures, and q_P being increased for this cell line when treated with both Buthionine Sulfoximine (BSO) and ME. By Day6, however, there were no significant differences between the cultures. Most interestingly, when treated with H_2O_2, the transiently transfected ECL cultures had a lower productivity in comparison with the other cell lines, despite this being the chemical that was used for evolution.

Stable pools were generated using the ECL and C2A cell lines (ECL_{SP}, $C2A_{SP}$) and subsequent producing cell lines were cultured in fed-batch and batch processes. In a fed-batch process, there were substantial and significant improvements in growth seen in the ECL_{SP} cell line in comparison with $C2A_{SP}$ throughout culture, as well as decreased ROS content at Mid- and Late-stage culture. Productivity was largely unchanged, with only a slight increase in ECLSP q_P in the Early stages of culture. In a batch process, the novel producer cell lines were treated with BSO, ME and H_2O_2, as with the transiently transfected cells seen previously. ECL_{SP} cultures appeared able to maintain their growth advantage over $C2A_{SP}$ cultures even when treated with these chemicals, with an increase in [rP] and q_P also seen in those cultures treated with BSO and H_2O_2. There was no significant difference in the productivity of ECL_{SP} and $C2A_{SP}$ cell lines treated with ME.

Chapter 8:

Discussion

Contents

List of Abbreviations

CHO	Chinese Hamster Ovary	DoE	Design of Experiments
Cu	Copper	[rP]	Product Titre
NAC	N-Acetylcysteine	TCA	Tricarboxylic Acid
MT	MitoTEMPOL	DHLA	Dihydrolipoic Acid
AA	Ascorbic Acid	MAb	Monoclonal Antibody
MQ	MitoQ	Se	Selenium
BHA	Butylated Hydroxyanisole	H_2O_2	Hydrogen Peroxide
SS	Sodium Selenite	ECL/ECL_{SP}	Evolved Cell Line
GSH	Glutathione	ME	Menadione
$IVCD_{MAX}$	Maximum Integral Viable Cell Density	BSO	Buthionine Sulfoximine
ROS	Reactive Oxygen Species	C2A/$C2A_{SP}$	Original Host Cell Line
q_P	Cell Specific Productivity	GCL	Glutamate Cysteine Ligase
LCA	L-Carnosine	VCD	Viable Cell Density
OTZ	Procysteine	CMO	Contract Manufacturing Organisation
AL	α-lipoic Acid	OXPHOS	Oxidative Phosphorylation

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8.1 Introduction

The preceding results chapters detail a body of work attempting to understand and manipulate the oxidative metabolism of industrially relevant host and producer CHO cells, in an attempt to improve the growth and/or production phenotypes of these cell lines. It has been demonstrated that oxidative stress impacts productivity and that cell line characteristics can be altered through media supplementation of pro-oxidant and anti-oxidant chemicals during the production culture process and through directed evolution strategies utilising chronic exposure to oxidative stress.

Understanding the data presented is critical to moving forward in any research endeavour, and so the following pages will attempt to extricate meaning from the patterns and trends described previously, through an understanding of the scientific mechanisms and existing literature.

8.2 Media Composition and Its Role in the Lactate Switch

Investigation into the literature, as discussed in Chapter 2, indicates that media formulation is just as important in a process development strategy as cell line engineering. Appropriate media will contain an adequate carbon source, along with amino acids, essential minerals and vitamins and traces of certain metals (Ling *et al*, 2015). It is clear from the characterisation studies of host cell line C2A and producer cell lines 7C3 and 6A8 that the CHO cells used in this study exhibit a metabolic switch during the stationary phase of their growth from lactate production to lactate consumption. Due to the continued feeding conducted through the cell culture period, this lactate consumption is not a survival mechanism due to glucose deficiency in the growth media. Studies by Luo *et al* (2012) indicate that this switch is elicited in producer cell lines only when there is a high enough concentration of copper (Cu) in the growth media. Copper is essential for the function of many enzymes within the cell, including cytochrome C oxidase IV in the electron transport chain, and CuZn-Superoxide Dismutase (Gaetke & Chow, 2003), making its presence in media crucial in terms of the oxidative metabolism of the cell. Cells without enough copper in their environment are unable to utilise oxygen efficiently due to impaired oxidative phosphorylation, and the subsequent mitochondrial damage would mean that lactate dehydrogenase is unable to metabolise any lactate generated through glycolysis (Luo *et al*, 2012).

It stands to reason, then, that there is a reasonably high concentration of Cu in Gibco® CD OptiCHO™ (Thermo Fisher Scientific, Paisley, UK), the commercially available media used to grow producers 7C3 an 6A8. While it was postulated that there was no lactate switch seen in the host cell line C2A due to the brevity of the culture period, it is also possible that the growth media used in its culture period (Gibco® CD DG44, Thermo Fisher Scientific) also has a lower Cu concentration, meaning the cells do not have the oxidative capacity to metabolise lactate. It is useful to note that

Cu is, as with most media additives in cell culture processes, somewhat of a double edged sword. While it provides support to a variety of key cellular enzymes, an overabundance of the metal is toxic to cultured cells through induction of oxidative stress (Yuk *et al*, 2014). In the presence of superoxide, such as that produced through electron leakage during oxidative phosphorylation or fatty acid oxidation, or when exposed to reducing agents such as glutathione, Cu^{2+} ions can be reduced to Cu^{+}, which is then able to go on and catalyse the creation of hydroxyl radicals from hydrogen peroxide through the Haber-Weiss reaction (Eq. 8.1 and 8.2) (Gaetke *et al,* 2003).

$$*O_2^- + Cu^{2+} \rightarrow O_2 + Cu^+ \qquad \textbf{Equation 8.1}$$

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^- + OH^* \qquad \textbf{Equation 8.2}$$

8.3 Exploring the Most Effective Anti-Oxidant Feeding Strategies

8.3.1 Patterns Identified in Batch Culture Screening

At first glance the responses of producer cell lines 7C3 and 6A8 to anti-oxidant treatment in a batch process appear cell line specific; N-Acetylcysteine (NAC), MitoTEMPOL (MT) and Ascorbic Acid (AA), for example, elicit positive changes to the productivity phenotype of 7C3 cells cultures, whereas MitoQ (MQ), Butylated Hydroxyanisole (BHA) and Sodium Selenite (SS) do so in 6A8 cultures. There are, however, clear trends in both mechanism and timing that identify the type of anti-oxidant chemical that may be used in successful feeding strategies.

8.3.1.1 Introducing Anti-Oxidants Pre-Exponential Growth

Glutathione (GSH) consistently elicited increases in $IVCD_{MAX}$ while reducing cellular Reactive Oxygen Species (ROS) content in both 7C3 and 6A8 cell lines when included in the growth media at the point of inoculation. There was no correlating increase in productivity in these cultures, and in fact although overall titre was higher, cell specific productivity (q_P) of 7C3 cultures was slightly decreased, suggesting that the extra support provided to the glutathione pool early on in culture had a direct impact on the cells' ability to accumulate biomass but that this oxidative protection did not extend long enough into the culture period to impact protein folding and secretion. In contrast, when introduced to cell culture at the end of exponential growth, GSH was still able to reduce ROS levels in both cell lines, but this no longer resulted in an increase in $IVCD_{MAX}$, nor did it translate to an increase in productivity. Another compound that specifically elicited a positive response to both cell lines when introduced at the beginning of culture was L-carnosine (LCA), a scavenging compound that is known for its powerful Cu-chelating mechanism. Introduction of LCA on Day0 of culture resulted in an increase in $IVCD_{MAX}$ in both 7C3 and 6A8 cultures, as well as an increase in overall product titre in 7C3 cultures. The addition of this compound, however, while not impacting the ROS

content of cells earlier in culture, did result in an increase in overall cellular ROS content in the later stages of culture.

Mitochondrially targeted anti-oxidants, as discussed previously, are potentially very powerful tools, sending ROS-neutralising agents directly to one of the main sources, and main victim of ROS within the cell. In batch cultures, both cell lines saw a decrease in late stage ROS content and an increase in $IVCD_{MAX}$ when treated with a mitochondrially targeted anti-oxidant at the point of inoculation, but different ones were effective for the different cell lines. In 7C3 cultures, MT decreased ROS in the early stages of culture and resulted in an increase of $IVCD_{MAX}$, but MQ was the more effective compound, with a ROS decrease in MQ-treated 7C3 cultures later in culture along with an increase in $IVCD_{MAX}$. In 6A8, MQ had no impact on cellular ROS, growth or productivity at any stage in culture. MT, however, was able to reduce ROS levels at both early and late stationary phases of growth as well as increase $IVCD_{MAX}$.

8.3.1.2 Introducing Anti-Oxidants Post-Exponential Growth

Response to anti-oxidants introduced at 96 h was much more muted than that when introduced at the point of inoculation. When introduced at the end of exponential growth, many more anti-oxidant compounds were able to elicit a reduction in cellular ROS than when introduced on Day0 of culture, however these ROS reductions were less likely to correlate with IVCD or productivity increases as those seen in cultures treated earlier. For example, there were only increases in IVCD seen in 7C3 cultures treated at 96 h by BHA, LCA, SS and Procysteine (OTZ), and only cultures treated with BHA, MQ and SS saw increases in product titre, despite overall decreases in ROS seen in those cultures treated with AA, BHA, MT, AL, GSH, NAC and OTZ. Similarly, only 6A8 cultures treated at 96 h with AA and SS saw increases in IVCD, and those treated with MQ saw increases in product titre and q_P, despite decreases in cellular ROS in cultures treated with BHA, GSH, MQ, SS and α-lipoic acid (AL).

As with those cultures treated on Day0, however, there were similarities seen between the cell lines with respect to anti-oxidant chemicals that improved their growth or productivity characteristics. When both 7C3 and 6A8 cell lines were treated with BHA, late stage ROS was reduced and final day product titre was increased, with $IVCD_{MAX}$ also increased in 7C3 cultures. SS was also able to increase growth and product titre in both cell lines, with a decrease in 6A8 cellular ROS levels.

7C3 cultures treated on Day4 of culture were not responsive to mitochondrially targeted anti-oxidants, despite both MQ and MT having a positive impact on growth and cellular ROS levels of this cell line when introduced at the point of inoculation. When introduced on Day0 of culture, MT was able to increase growth and product titre in 6A8 cell lines, as well as decreasing cellular ROS. It

was MQ, however, that was able to improve ROS levels and productivity of the same cell line when anti-oxidants were introduced on Day4. Despite the two compounds being similar in design and mechanism, this implies that these cell lines do not respond well to MT when it is introduced later in culture and that its main benefits come when included at the pre-exponential stage of culture. This difference in response is likely due to the mode of action of these mitochondrially targeted chemicals along with the metabolic states of the cells; Oxidative Phosphorylation is at its highest during the exponential phase of growth as the cells are generating energy for growth and proliferation. It is therefore during this period of growth that the mitochondrial contribution to the cellular oxidative burden is at its highest: introducing an anti-oxidant chemical at the beginning of this process to target the ROS leaking during OXPHOS will have most effect then. During stationary phase of growth, metabolic flux through the mitochondria is much reduced and the source of ROS will have shifted to other areas of the cell, such as the Endoplasmic Reticulum.

MitoTEMPOL is the more recently developed of the two synthetic anti-oxidants, and while there has been extensive characterisation of MT conducted by groups such as Murphy *et al* (2009), MQ remains the most well characterised of the two in animal models of oxidative stress and disease (Li *et al,* 2013) and there are currently no bodies of work that have been conducted comparing the efficacy of the two and so it is difficult to see if this difference in response is typical.

Also a promising compound when introduced at the beginning of culture, L-carnosine was far less effective when introduced after exponential growth. The scavenging compound elicited no positive impact on the growth, productivity or cellular ROS levels of 6A8 cell line, and while there was an increase in 7C3 culture $IVCD_{MAX}$, this actually correlated with an increase in cellular ROS levels, indicating that supplementation with LCA is more appropriate before exponential growth.

8.3.2 Fed-batch Screening

Predictions from the DoE statistical modelling generated by G. Ninon (FUJIFILM UK, Process Design Group) supported the theory that anti-oxidant compounds with specific mechanisms are most likely to elicit a positive change in growth and productivity characteristics of a CHO cell line when introduced at specific points in culture, and not at other time points. While a useful indicative tool for designing fed-batch feeding strategies, it should be noted that there is a distinct difference between introducing chemicals at the mid-point of a batch culture, and of introducing chemicals at the point of feeding in a fed-batch culture. Cells in a fed-batch culture environment display different metabolic characteristics to those in a batch culture; for example, there was no lactate switch in batch cultures but there was in fed-batch cultures, likely due to the truncated culture period, however this metabolic difference alone would likely have a significant impact on the way the cell

lines react to a perturbation of their redox status. Despite this experimental shortcoming, the predictions produced from the batch-culture data provided feeding strategies of interest. Mechanistic similarities were identified while specific anti-oxidants differed for the individual cell lines (such as MT for 7C3 and MQ for 6A8). While responses to anti-oxidant chemicals were cell line specific in nature, however, the characteristics displayed in response to fed-batch feeding strategies showed similarities between cell lines; producer cell line APC had not been tested in batch conditions, but also displayed similar characteristics to 7C3 and 6A8.

Feeding strategies containing LCA, GSH or a combination of LCA and GSH in the basal growth media were consistently able to increase $IVCD_{MAX}$ in all three cell lines in at least one strategy, while supplementation of feeding media with AL also correlated with an increase in growth in 7C3 and APC producer cell lines. AL and GSH represent the two most effective scavenging anti-oxidants trialled, possibly known, with their redox couples allowing for strong scavenging abilities throughout the ROS and RNS molecular families. LCA is also a scavenging agent, but its predominant anti-oxidant capacity lies in its chelating activity of Cu^{2+} ions. The mitochondrially targeted compounds did not perform well in this fed-batch trial, rarely positively impacting the growth or productivity of the cell lines trialled. Where there were improvements it was often in terms of growth, and at the expense of productivity. For example, feeding strategy B supplemented the feeding media for cell line 7C3 with MitoTEMPOL which lead to an increase in $IVCD_{MAX}$ and a decrease in late-stage cellular ROS content, but also a drop in [rP] and q_P. Similarly, feeding strategies R and S supplemented the feeding media for APC cultures with MT and MitoQ, respectively, and were able to significantly increase $IVCD_{MAX}$ while at the same time not altering q_P, and actually increasing cellular ROS content. The same could be said of anti-oxidant compounds that provide enzymatic support: 7C3 cultures with N-Acetylcysteine in their feed and APC cultures with Sodium Selenite in their feed (F and O, respectively) saw increases in their $IVCD_{MAX}$ but no improvement in their productivity, with F actually seeing both decreased titre and q_P.

It is odd that there was so little response at all from the 6A8 cell line in the first fed-batch trials. Cell cultures treated with feeding strategy G (GSH in basal media and BHA added to feed) saw an improvement in both $IVCD_{MAX}$ and late stage ROS content, but otherwise the responses to any of the anti-oxidant feeding strategies trialled with this cell line were often either negative (in terms of growth), or insignificant. Despite the more prominent responses seen in batch experimentation, it appears the DoE modelling and interpretation was ineffective in isolating compounds that would be effective for this cell line.

8.3.3 Feeding Strategy β: One Size Fits Most?

The one feeding strategy identified that improved all three cell lines was feeding strategy β, whereby L-carnosine was introduced in the basal growth media and α-lipoic acid was included in the feeding media. While LCA is a scavenging anti-oxidant, its predominant benefit in terms of oxidative metabolism lies in its copper chelation activities (Kohen *et al,* 1988). A higher copper concentration in culture media in general is beneficial, a low Cu concentration leads to consistent lactate production which is associated with a decreased TCA cycle capacity due to the mitochondrial damage associated with the corresponding increase in oxidative stress (Luo *et al*, 2012). However, this benefit from copper concentration is only seen at the point in culture when the switch to lactate consumption occurs. In 6A8 and 7C3 cultures, this switch was seen at 148 h (Chapter 4, "*Characterisation of Cell Lines*", Figure 4.4, pg62).

High copper concentration in cell culture can lead to an increase in cellular ROS levels when exposed to reducing agents such as glutathione, catalysing the conversion of Hydrogen Peroxide to Hydroxyl Radicals via the Haber-Weiss reaction (Gaetke & Chow, 2003). Earlier in culture, the glutathione pool has not been depleted by excess ROS generated in processes such as Oxidative Phosphorylation and protein folding and secretion, and so cells are more susceptible to Cu cytotoxicity, meaning that Cu chelation from LCA is far more beneficial to the cells. As the cells switch from Warburg metabolism to favouring oxidative phosphorylation, it is more beneficial for cell activity to have higher environmental Cu concentrations, hence the unfavourable responses seen in the batch culture screening when LCA was added after exponential growth.

α-lipoic acid, along with its reduced version dihydrolipoic acid (DHLA), is a powerful anti-oxidant agent, and when acting synergistically as a redox couple, AL/DHLA are able to neutralise most ROS and RNS produced in the cell. Included in the feeding medium, AL was introduced to the cell cultures on at 72 h, 120 h, 168 h and 240 h of culture, and is therefore able to provide a consistent oxidative buffer as the culture progresses away from Warburg metabolism and into oxidative phosphorylation as the main source of energy, and further as protein production increases during the stationary phase of growth.

In these cell lines, there were only improvements in growth seen in cultures treated with feeding strategy β; while there were increases in final day product titre seen in cell line 6A8, this was due to the extended culture growth period, rather than an increase in cell specific productivity. The recombinant MAb that all three cell lines are producing, however, is an easy to produce MAb protein that is less of an oxidative burden on the cells than emerging, difficult to express non-MAb biologics may be due to the increased incidence of phenomenon such as the UPR. It is very likely that such a feeding strategy, if employed when cell lines were producing such a biologic, would enable higher titres than if no such anti-oxidant feeding strategy were employed.

8.3.4 Alternative Promising Feeding Strategies and Their Properties

Feeding strategy β was able to consistently improve growth and cellular ROS content across all three cell lines, but was not the only strategy that showed promise for improving cell activity. Feeding strategy α, whereby LCA was included in the basal growth media and MT was included in the feeding media, was able to reduce late stage cellular ROS in producer cell lines 7C3 and 6A8 and increase growth across all three cell lines. As with β, there was an increase in product titre in 6A8 due to the extended growth period compared to untreated cultures. As discussed when exploring β, LCA's copper chelating activity during exponential phase is likely to protect cells from copper toxicity while they recover from the bottleneck population experienced after seeding. As cells move from exponential growth to stationary, there is a metabolic shift and the level of oxidative phosphorylation becomes higher, and mitochondrially targeted anti-oxidants such as MT can therefore provide ROS neutralisation at the primary ROS production site.

The other two feeding strategies that improved the characteristics of producer cell lines tested were variants on a combination of using a scavenging compounds in the basal media and enzymatic support in the feeding media. Feeding strategy ε combined the powerful scavenging anti-oxidant AL in the basal media, with Sodium Selenite, the soluble form of Selenium (Se). Se is an essential component in such anti-oxidant enzymes as Glutathione Peroxidase, Thioredoxin Reductase, and Iodothyronine Deiodinases (Tinggi, 2008), and the combination of supplementation with this essential trace element in the feeding media and addition of a powerful scavenger in the basal media was able to reduce late stage cellular ROS in 6A8 cells, increase $IVCD_{MAX}$ of APC cells and increase q_P of 7C3 cells. While not as powerful a scavenger as AL, when AA was included in the basal media combined with NAC, a cysteine precursor to support glutathione synthesis, late stage cellular ROS levels were reduced and $IVCD_{MAX}$ was increased in APC cultures in comparison with their untreated counterparts.

8.4 The Improved Characteristics of the Evolved Cell Line

In an attempt to engineer a cell line more resistant to oxidative stress, an industrially relevant host cell line was chronically exposed to Hydrogen Peroxide (H_2O_2), a broad acting pro-oxidant chemical, for a period of roughly 130 generations. H_2O_2 ensured an increased level of ROS in the cellular environment, ensuring only cells with increased anti-oxidant defences or lower intracellular ROS production would survive. Since the host cell line had not been transfected with the gene for a recombinant product, the evolution characteristic chosen to determine increased resistance was the growth and viability of the cells, with acceptable adaptation levels designated to be viable cell density recovery to within standard error of the viable cell density of untreated cells, or percentage

viability to be above 80 %. Cells were subcultured every 72 h with fresh H_2O_2 added each subculture, meaning that it was early stage growth that was the determining factor in the evolution process.

8.4.1 Nature of the Oxidative Resistance

Evolved cell lines post transient transfection (ECL), as well as those isolated from stable transfection pools (ECL_{SP}), were stressed with specific pro-oxidant chemicals designed to encourage redox cycling (Menadione, ME), inhibit glutathione synthesis (Buthionine Sulfoximine, BSO) and raise the overall cellular ROS content (Hydrogen Peroxide, H_2O_2). In both cases, the cell cultures exhibited similar responses to the chemicals in comparison to the original non-evolved cell line (C2A or $C2A_{SP}$).

When the levels of cellular ROS were artificially increased through redox cycling, q_P was only marginally higher in ECL than C2A, and when ROS levels were increased through direct supplementation with H_2O_2 ECL q_P was decreased in comparison with C2A, despite this being the chemical that was used in the evolution process. However, when the cells' ability to defend against oxidative stress was compromised through inhibition of Glutamate Cysteine Ligase (GCL), ECL q_P was increased in comparison to C2A and also in comparison to q_P of untreated ECL. In batch cultures of ECL_{SP} and $C2A_{SP}$ cells, $IVCD_{MAX}$ was severely impacted after BSO treatment. ECL_{SP} productivity, however, was not, with overall titre not significantly decreased in comparison to untreated cells and q_P thus significantly increased. Introducing ME at the point of inoculum did not impact the final day titre of either cell line, and although $IVCD_{MAX}$ was negatively affected in both cell lines, there was less of an impact in ECL_{SP} and they maintained a significant advantage over $C2A_{SP}$. Unlike the transiently transfected cells, H_2O_2-treated ECL_{SP} continued to out-perform $C2A_{SP}$. However, like the transiently transfected cells, ECL_{SP} did not display the kind of resistance to H_2O_2 treatment that the cells achieved during the evolution process.

While in general, ECL and ECL_{SP} outperform C2A and $C2A_{SP}$ in standard post-transfection culture processes and batch and fed-batch culture processes, whether artificially stressed or not, they are not completely resistant to all forms of oxidative stress. Indeed, they do not maintain the level of resistance to H_2O_2 developed during the evolution process. In work done by Spitz *et al* developing oxidative-stress resistant Chinese Hamster fibroblasts in 1988, loss of H_2O_2 resistance was also seen after 60 passages post removal of H_2O_2. ECL/ECL_{SP} was not subcultured as much post H_2O_2 removal before loss of resistance, however the process of transfection is highly stressful and this could have accelerated the instability of the cell line. It does appear, however, that the ECL/ECL_{SP} cells are better able to respond when the oxidative defences are compromised rather than when ROS levels increase. These results, combined with the decreased ROS levels seen in untreated fed-

batch ECL_{SP} compared to $C2A_{SP}$ suggest that the mechanism of evolution was through a reduction in cellular ROS production rather than an increase in defence activity, such as seen by Spitz *et al* (1998).

8.4.2 Potential for Manufacture Scale-Up

Improvements were seen in the growth of evolved cell lines throughout the culture period in both a batch and a fed-batch process. Particularly of interest, however, is the improvement seen in exponential growth. Figure 8.1 shows the workflow for the typical manufacture scale up process from a 30mL shake flask culture to a 2000L production scale bioreactor. Typically, between each expansion phase, cells will be grown for 4 days to allow for 3.3 population doublings, ensuring that a 1:10 dilution will be feasible.

Figure 8.1. Workflow schematic for culture expansions during the manufacture scale-up process. Cells are allowed to grow for 4 days between each expansion, projected time scale from 30 mL to 2000 L is 24 days.

The Viable Cell Density (VCD) and population doublings after three days of growth for ECL_{SP} and $C2A_{SP}$ are shown in Figure 8.2. Growth of ECL_{SP} was significantly higher than $C2A_{SP}$, with a calculated reduction of cell doubling time from 26 h to 23 h. Such a reduction in doubling time enabled the evolved cells to reach the required number of population doublings one day sooner than stable cells isolated from the original host cell line. The projected timescale from 30 mL to 2000 L using the original cell lines accounting for 4 days growth between each volume expansion is 24 days. However, with the novel ECL_{SP} growing for 3 days between each volume expansion this projected timescale would be reduced to 18 days, a reduction of just under a week. For a CMO such as FUJIFILM, a reduction of the manufacture scale-up timescale of a week would enable up to 3 extra production runs per annum, a cell line improvement that would therefore provide a significant financial incentive for further development.

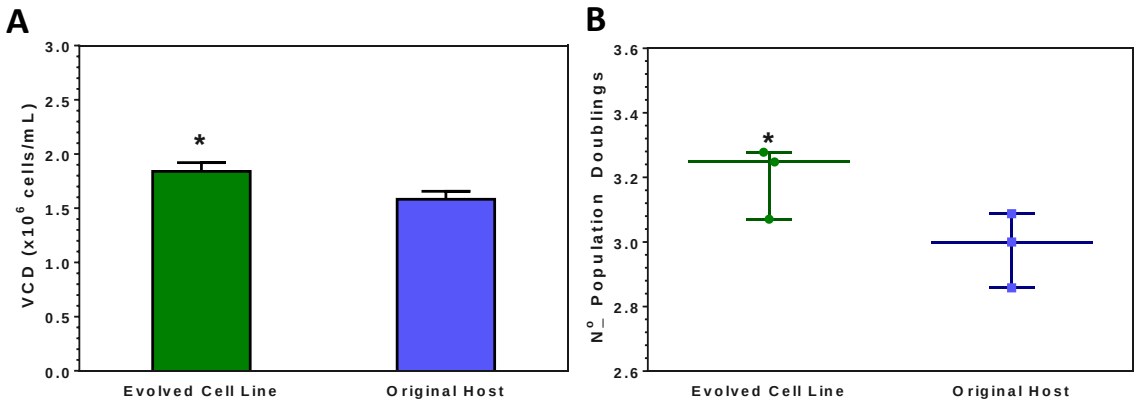

Figure 8.2. (A) Viable Cell Density of ECL_{SP} and $C2A_{SP}$ after 3 days of growth. Data bars indicate Mean average of triplicate cultures with error bars indicating Standard Error of the Mean (B) Number of Population Doublings achieved by ECL_{SP} and $C2A_{SP}$ within 3 days of growth. Triplicate samples are indicated by individual data points. * indicates significant difference of ECL_{SP} compared to $C2A_{SP}$, $p<0.1$, generated using GraphPad Prism.

Chapter Summary

When treating producer cell lines 7C3, 6A8 and APC with anti-oxidants there were clear benefits to ensuring compounds with certain mechanisms were introduced at specific times in cell culture. Strong chelating activity such as seen in L-carnosine elicited a positive impact on $IVCD_{MAX}$ and late stage ROS levels when introduced at the beginning of culture, but when introduced after exponential growth did not produce any benefit. Unsurprisingly, the powerful anti-oxidant compounds glutathione and α-lipoic acid both contributed to decreased cellular ROS levels and increased growth, but glutathione provided the most benefit when introduced pre-exponential growth rather than after.

Most of the benefits seen in anti-oxidant supplementation were in terms of growth, rather than cell specific productivity. During cell line and process development, it is often difficult to improve growth without it being at the expense of productivity, or vice versa (Wurm, 2004), but this response to anti-oxidant supplementation suggests that OXPHOS and fatty acid oxidation rather than protein folding and secretion are the main sources of oxidative stress in these cell lines when producing this recombinant protein. It is possible that the anti-oxidant supplementation would be beneficial in improving product titre and q_P if the cell lines were producing a difficult to produce, non-MAb biologic.

Chronic exposure to increasing concentrations of H_2O_2 for 130 generations created an oxidative stress resistant host cell line that, when transfected with a recombinant MAb, was able to maintain resistance and had improved growth and productivity through both transient and stable transfection processes. Probing of the novel cell line with pro-oxidant chemicals in addition to fluorescent staining for cellular ROS levels suggested that the nature of the oxidative resistance lies

in a reduced cellular production of ROS rather than increased defence mechanisms. Doubling time of the evolved cell line was reduced from 26 h to 23 h and the required number of population doublings for volume expansion during manufacture scale up was therefore achieved a day earlier than the original cell line, potentially meaning the manufacture scale-up timescale could be reduced by up to a week.

Chapter 9:

Conclusions and Thoughts on Future Work

Contents

List of Abbreviations

ROS	Reactive Oxygen Species	**SOD**	Superoxide Dismutase
MAb/rMAb	Monoclonal Antibody/Recombinant	**dO_2**	Dissolved Oxygen
$IVCD_{MAX}$	Maximum Integral Viable Cell Density	**H_2O_2**	Hydrogen Peroxide
FAO	Fatty Acid Oxidation	**GCL**	Glutamate Cysteine Ligase
OXPHOS	Oxidative Phosphorylation	**CMO**	Contract Manufacturing Organisation
TCA	Tricarboxylic Acid	**rProteins**	Recombinant Proteins
PCR	Polymerase Chain Reaction		

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9.1 Anti-Oxidant Supplementation to Improve Producer Cell Characteristics

A series of iterative rounds of screening with anti-oxidant chemicals led to the identification of an anti-oxidant supplementation strategy for fed-batch culture processes of producer cell lines APC, 7C3 and 6A8 that reduced cellular ROS content and increased overall growth. While responses to anti-oxidant supplementation in a batch process appeared to be cell line specific, there were mechanistic similarities between the anti-oxidant compounds that elicited a positive response, and ultimately the close lineage of the three cell lines meant that a single feeding strategy employing early stage copper chelation followed by feeding with a powerful scavenging agent was identified. In all cases, improvements in growth correlated with static or decreased productivity, a phenomenon common when developing novel processes (Wurm, 2004). Improvements in growth when ROS levels are decreased suggest that processes involved in biomass accumulation such as fatty acid oxidation and oxidative phosphorylation were the main causes, and targets of oxidative stress in these cell lines when producing an easy-to-express recombinant MAb.

9.1.1 Investigations into Cell Line Variation

Through various rounds of cloning and stable transfection, all three producer cell lines originate from the same DG44 CHO progenitor. As can be seen by even a cursory glance at their growth and productivity characteristics, there are large variations between the performance of cells developed from cell line types such as CHO-DG44, CHO DUKX-B11, CHO-S and CHO-K1SV, not to mention individual cell lines developed by biomanufacturing companies for specific productivity needs. Given the variation between cell and tissue types seen in medical research investigating anti-oxidant compounds, it is highly likely that different cell lines will have different responses to anti-oxidant treatment. Since the feeding strategies identified employ well-characterised, powerful anti-oxidants (L-carnosine, Glutathione, α-lipoic Acid, MitoTEMPOL) it is doubtful that they would not affect different cell lines at all, however it would be interesting to see if the same combination of compounds in basal and feeding media elicited a similar response (i.e., a decrease in late-stage ROS production and an increase in $IVCD_{MAX}$), or altered the cell lines in another way (i.e. altered product titre, cell specific productivity, early- and mid-stage ROS production).

High-throughput plate-based screening is a potentially powerful tool that can be used to substantially decrease the time-scale for compound screening in media development, and systems such as the Deutz (Applikon® Biotechnology, California, USA) have exploited and advanced such technologies. Recent successes in this area include the development of ValitaQC for media screening and other plate-based assays by Ben Thompson and Valitacell. The plate based screening utilised in this study was able to identify appropriate concentrations for use in anti-oxidant treatment in terms

of growth but was unable to predict which compounds would be most beneficial in shaking culture processes. The screening of anti-oxidants in batch and fed-batch cultures, while effective, meant running dozens of cultures simultaneously for several months and was both highly time and resource consuming. Further development of a high-throughput platform to more reliably predict cell line response to anti-oxidants, perhaps using alternative identifiers to growth such as staining for ROS levels or key metabolites, would substantially decrease the cost of good associated with the process and enable developers to rapidly screen different cell lines with each anti-oxidant supplementation protocol. Use of deep-well plates and plate-shaking platforms could also enable the simulation of a fed-batch process using much reduced culture and feeding volumes, while also providing increased reliability when predicting performance in a fed-batch shaking culture process than using performance readings after 3 days growth in a static culture process.

9.1.2 Mechanistic Understanding and Further Process Development

Analysis of cell lines throughout this study has focussed on key indicators critical for researchers during process and cell line development: growth and productivity after treatment. While ROS levels were consistently monitored, a deeper understanding of the metabolic changes elicited by beneficial feeding strategies would help to confirm theories about why these particular anti-oxidant combinations were advantageous, and also aid prediction of how beneficial they would be when applied to different cell lines, or the same cell lines transfected with difficult-to-express non-MAb biologics. For example, a more thorough understanding of the changes in flux through the glycolytic pathway and mitochondrial activity (Fig. 9.1) at different stages in culture would elucidate whether anti-oxidant treatment altered the lactate consumption phenotype seen in untreated cultures; the

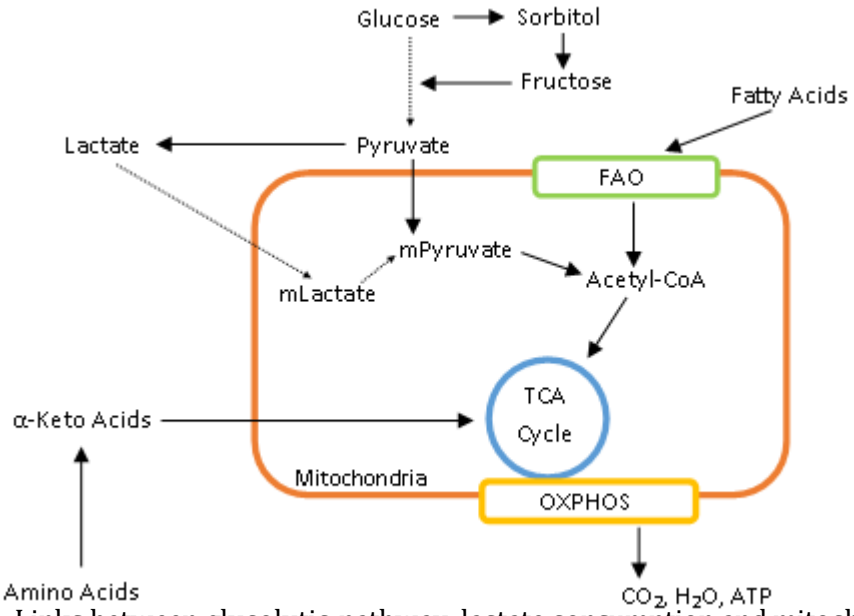

Figure 9.1. Links between glycolytic pathway, lactate consumption and mitochondrial capacity. The mechanisms surrounding the metabolic switch from lactate production to lactate consumption (dashed line) are still poorly understood, and a more thorough understanding of this metabolic flux may aid cell line engineering strategies.

copper chelation activity of L-carnosine, while potentially advantageous early on in culture and leading to increased biomass accumulation, could have resulted in cells having a lactate production phenotype, a phenotype associated with lower production.

The variety of 'omics technologies available could be powerful tools for analysing any genomic, transcriptomic, proteomic and metabolic changes to proteins and pathways associated with the oxidative metabolism would be beneficial for assessing any changes to redox defence systems post anti-oxidant treatment. Broad scale transcriptomics such as those offered by The Genome Analysis Centre™ (TGAC) would allow for a deeper understanding of whole-cell transcriptomic changes and would ensure no unpredicted changes were missed; however, the tool is a brute force approach to a transcriptomic map that, at the time of writing, is poorly annotated for the purposes of CHO cell analysis and is an incredibly time-consuming process. A more targeted approach would be to use redox specific tools such as a custom Affymetrix array or the Qiagen CHO Cell Oxidative Stress PCR Array, the latter of which would provide information on the relative genomic levels of glutathione peroxidases, SODs, catalase and oxygen transporters (to name a few) in the various cell lines tested with the novel anti-oxidant feeding strategies, but would limit the surreptitious discovery of pathways linked to the altered redox status of the cell, and would also limit the ability to monitor other areas of interest such as protein folding and secretion.

In all process development strategies, scalability is key to ensure consistency once processes are expanded to a manufacture scale. Applying the anti-oxidant feeding strategies to cell lines cultured in a system such as the AMBR® 250 system would allow for a fully automated trial in a more representative system than shaking flask cultures. Additionally, this would enable assessment of the novel feeding strategy when cell cultures are being monitored for parameters not maintained in shaking flask cultures, such as dO_2 levels in culture.

9.2 Novel Cell Line Developed Through Directed Evolution

It has been shown that it is possible to generate a novel cell line with reduced ROS production and significantly increased growth capacity through chronic exposure to steadily increasing levels of Hydrogen Peroxide (H_2O_2), a broad-acting pro-oxidant chemical used in this instance to artificially raise the incidence of oxidative stress in cultured host CHO cells. The evolved cell line was able to maintain these characteristics after both transient transfection processes, and after generation of stable pools, despite losing some of their acquired specific resistance to the evolution pressure, H_2O_2.

9.2.1 Mechanistic Understanding

While the nature of the oxidative resistance displayed in the evolved cell line has been postulated to be due to a decreased production of overall cellular ROS levels rather than an increase in any of the cellular anti-oxidant defence systems, it would be interesting to investigate this further, to see if the resistance could have been developed in a more direct manner (such as through genetic engineering using gene targets identified through such investigation). An obvious route to explore would be to establish any changes to the levels of anti-oxidant enzymes. One route to do this would be through 'omics investigations as discussed previously, specifically targeting enzymes such as catalase, SODs, Glutamate Cysteine Ligase (GCL), Glutathione Peroxidase, and so on. Alternatively, a strategy already started in the described work would be to probe the cell lines with a wider panel of inhibitors of anti-oxidant enzymes: Buthionine Sulfoximine, an inhibitor of GCL, was already used to test the cellular response, but other chemicals include Aminotriazole to inhibit catalase (Bayliak *et* al, 2008) or Diethyldithiocarbamate, a SOD inhibitor (Dumay *et al,* 2005).

An alternative way to establish a more detailed picture of the redox status of these novel evolved cells would be through fluorescent imaging after tagging for specific Reactive Oxygen Species. There are various fluorescent probes available for different types of ROS. Utilisation of these along with tagging for mitochondria, endoplasmic reticulum and nuclei followed by imaging using fluorescent or confocal scanning microscopy would give a clearer picture of whether the decreased ROS levels seen in flow cytometric analysis was a generalised response to the evolution process, or was due to a reduction in a specific kind of ROS, or a reduction in ROS generation at a certain location within the cell.

9.2.2 Product Quality

After transient transfection, cell specific productivity in the evolved cell line was increased in comparison to the host cell line. After generation of stable pools, overall product titre and cell specific productivity was not increased but was maintained, even despite the increase in growth. Since the oxidative capacity of the cells appears to have been increased, and the recombinant MAb transfected into the cells was an easy-to-express product, it is possible that transfection with a difficult-to-express, non-MAb biologic would result in a more dramatic increase in productivity in comparison to the original host cell. Utilising the 96-well plate add on to the Lonza 4D-Nucleofector would enable high-throughput transfection of the evolved cell lines with biologics of various degrees of difficulty to express. Developing cell lines that enable a "plug-and-play" strategy with customers' products is essential for CMO biomanufacturers, and this would provide more information as to

whether this evolution approach can produce a cell line able to perform better with a range of different rProteins.

Any improvement in cell line characteristics is only valuable so long as they don't come at the expense of product quality; increased growth or titre is no good if the product cannot be used! Typically, biological potency and (sometimes) specificity assays must be performed on any biologic product to assess its quality. In addition, rMAbs are usually also assessed for their glycosylation patterns, correct glycosylation is essential to prevent an immunogenic response in the patient (van Beers & Bardor, 2012). Non-human glycosylation patterns that have been shown to elicit immune responses include galactose-α1,3-galactose, N-glycolylneuraminic acid and α1,3-fucose (van Beers & Bardor, 2012), and it is generally accepted that CHO cells do not produce these isoforms whereas murine cell lines do (van Beers & Bardor, 2012). There has currently been no work investigating the link between oxidative stress and appropriate glycosylation, but it stands to reason that healthier cells with lower incidences of UPR and oxidative stress would be less likely to produce incorrectly folded rProteins. Each company will have a panel of quality control assays that ensure the product retains potency and complies with regulatory restrictions. It would be necessary to conduct these with product from the novel evolved cell line (and with any cells being treated with the aforementioned anti-oxidant supplementation strategies) to ensure this newly acquired oxidative stress resistance has not deleteriously impacted the quality of the rProtein being produced.

9.3 Combining Engineering Strategies and Exploring Other Possibilities

The directed evolution of host cells represents an engineering strategy aimed at reducing oxidative stress at an early stage of cell line development, whereas the anti-oxidant feeding regimes represent a process engineering strategy aimed at achieving the same goal. Promising feeding strategies identified were developed in cell lines derived from the same host cell line utilised in the directed evolution methodology, and so it is probable that they would further improve the redox status of our novel evolved cell lines. Given that both strategies increased growth, however, it would be important to ensure that other areas of the production process were engineered appropriately to compensate for the increased biological material; high cell densities can lead to difficulties in clarifying production supernatant by depth filtration or centrifugation.

An engineering strategy not explored in this body of work is the artificial increasing of genes useful in oxidative resistance through either upregulation of existing genes or transfection with more copies of anti-oxidant enzymes. Previous studies show that over-expression of Catalase led to a less aggressive cancer phenotype (Glorieux *et al,* 2011) and a higher sensitivity to TNF-α induced apoptosis in HepG2 and MCF-7 cells (Bai & Cederbaum, 2000; Lüpertz *et al*, 2008). Over-expression

of GCL protected tumour cells against oxidative stress related death (Cortes-Wanstreet *et al*, 2009), while SOD2 over-expression in HeLa cells regulated radiation resistance and decreased radiation generation mito-ROS (Hosoki *et al*, 2012). In a more targeted approach, over-expression of mitochondrially targeted catalase protected Insulin-producing cells from ROX toxicity (Gurgul *et al*, 2004). The evidence from these studies, amongst several existing in the cancer research literature, suggest that another early-stage cell line development strategy that is likely to produce oxidative stress resistant cell line would be the over-expression of relevant anti-oxidant enzymes.

Concluding Remarks

The relationship between oxidative stress and productivity in CHO cell factories is an area of research in its infancy, with a renewed interest only recently following key papers demonstrating the importance of the redox status of cells producing rProteins. This body of work demonstrates the beginning steps of attempting to utilise knowledge of both oxidative stress, garnered largely in the medical research field, and the field's extensive experience with CHO cell culturing processes developed after more than 6 decades of research, to engineer oxidative stress resistance in industrially relevant CHO cells producing industrially relevant biologics. A clear link between a reduction in ROS levels and an improvement in cell line performance has been demonstrated, but more investigation is needed to unpick the precise mechanistic underpinnings of the resistance demonstrated as well as to attempt to address the imbalance of improvements in growth compared to productivity.

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A doctorate, whilst a solitary endeavour, is not one conducted in isolation, and it is with gladness that I now thank those people and institutions without whose support the completion of my studies would not have been possible.

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My successful completion of my doctoral studies was due in no small part to the unending patience and love of one man. I thank Dimitri for being my confidant, my comedian, my sounding board, my sparring partner, my companion. Finally, I thank my family, who never cease to amaze me with their love and support for me and my goals. Without Alex, Jack, Mum and Dad I would never have got to where I am today and for that I am eternally grateful.

Appendices

Appendix A: Flow Cytometry Gating Procedures

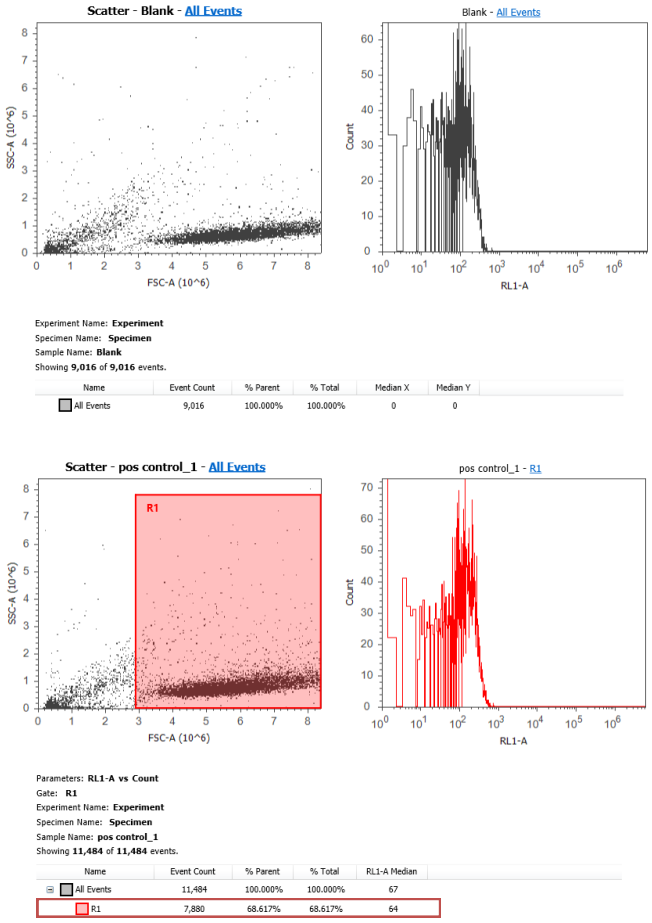

Figure AA.1. Gating procedure for CellROX stained cells when using the Attune Autosampler flow cytometer. Scatter plot graph was gated to exclude debris, and Median Fluorescence was taken from the Histogram for the RL1-A filter.

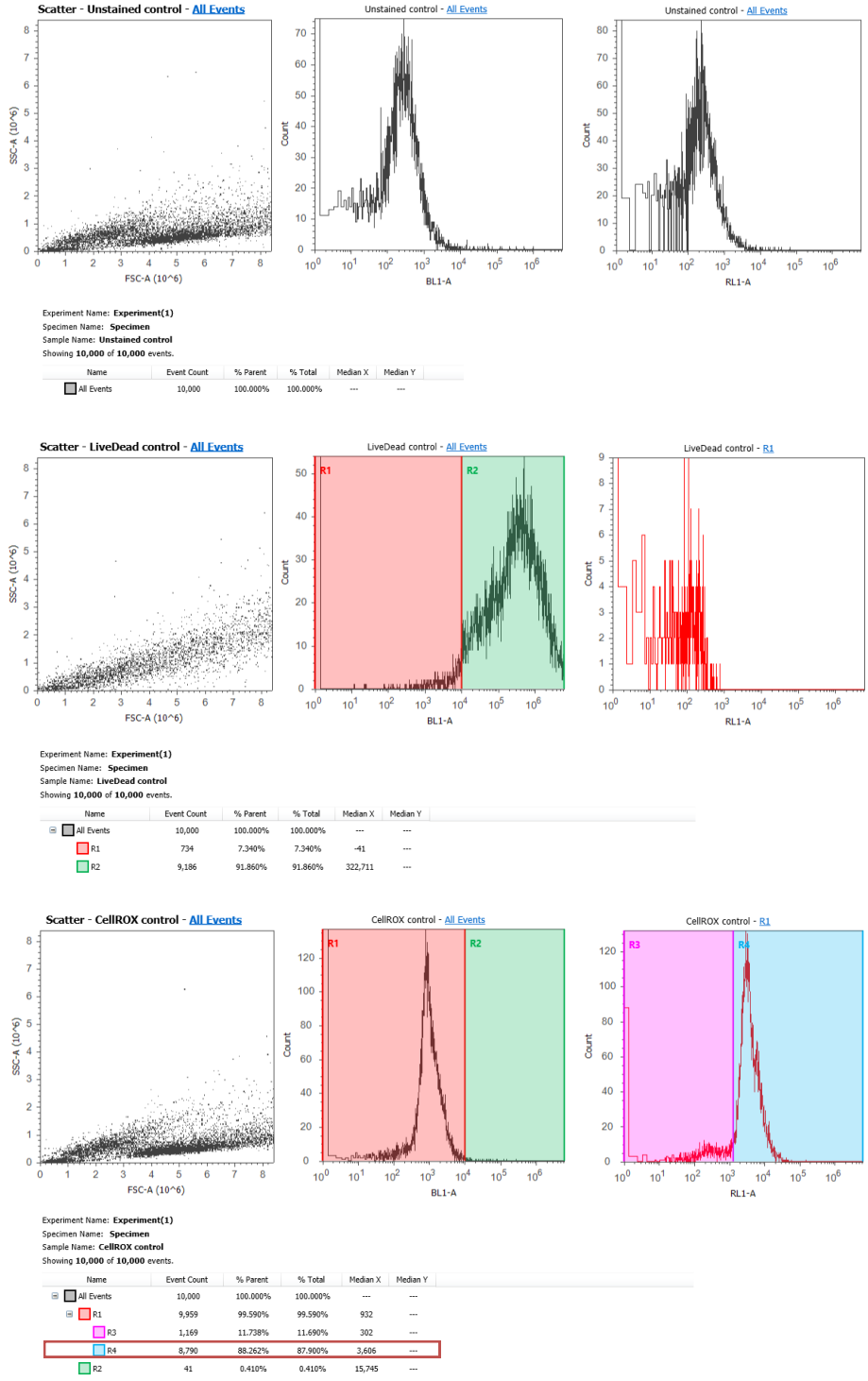

Figure AA.2. Gating procedure for LIVE/DEAD Fixable Green Dead Cell Stain and CellROX Deep Red stained cells when using the Attune Autosampler flow cytometer. Bi-marker gates were used in BL1-A Histogram of LIVE/DEAD positive cells to exclude dead cells. RL1-A Histogram was generated from the live cells (R1) and a Bi-Marker gate generated to exclude negative samples from CellROX stained cells. Median fluorescence of CellROX stained live cells was used as an indicator of ROS levels (R4).

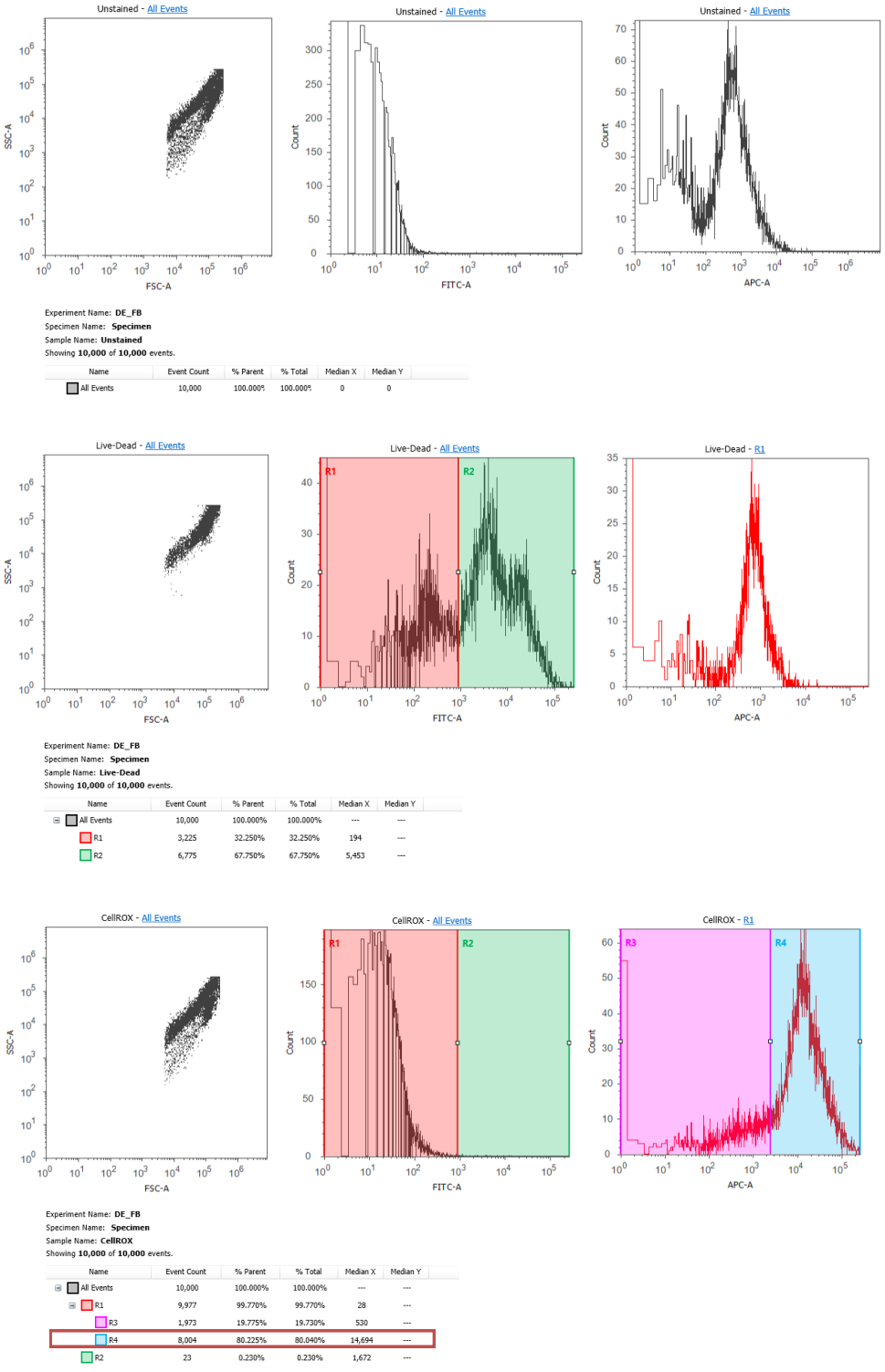

Figure AA.3. Gating procedure for LIVE/DEAD Fixable Green Dead Cell Stain and CellROX Deep Red stained cells when using the BD FACSAria II flow cytometer. Bi-marker gates were used in FITC-A Histogram of LIVE/DEAD positive cells to exclude dead cells. APC-A Histogram was generated from the live cells (R1) and a Bi-Marker gate generated to exclude negative samples from CellROX stained cells. Median fluorescence of CellROX stained live cells was used as an indicator of ROS levels (R4).

Appendix B: Dose Response Curves for Pro-Oxidants and Anti-Oxidants in Producer Cell Lines 7C3 and 6A8

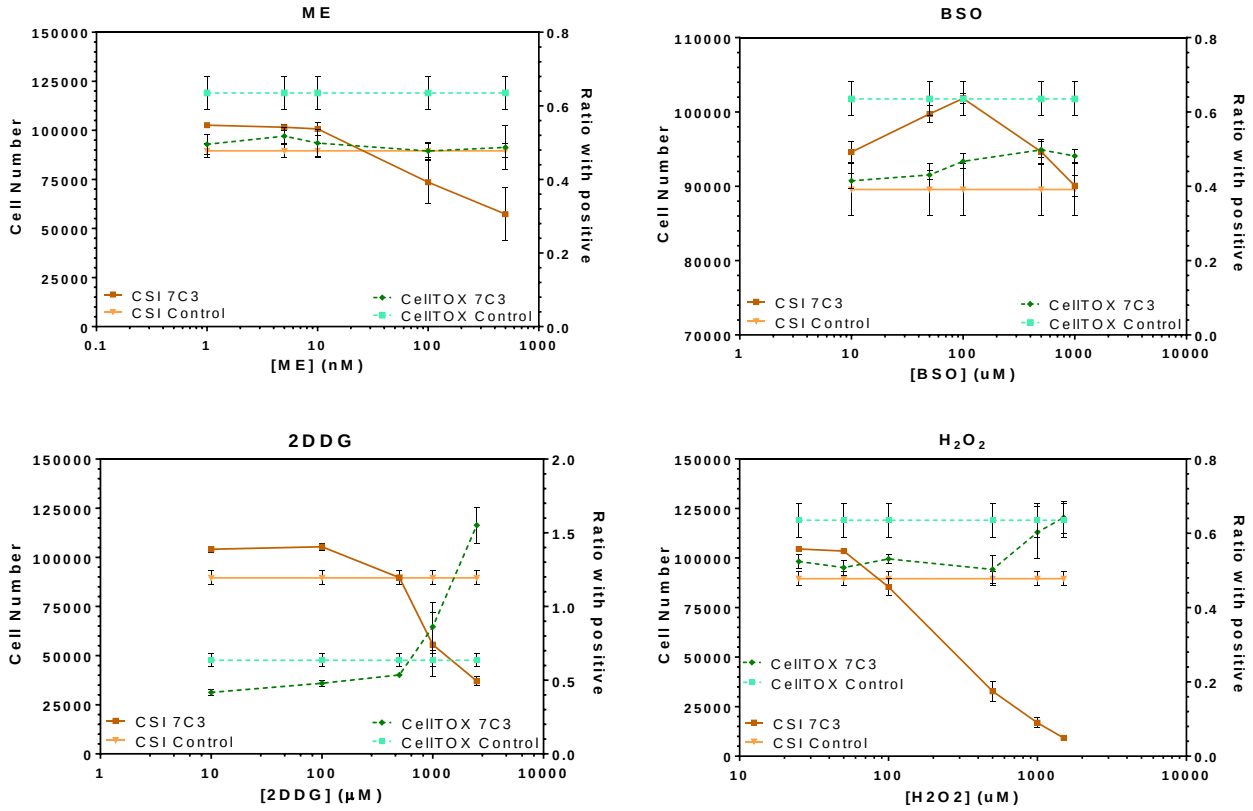

Figure AB.1. Dose Response curves for producer cell line 7C3 when treated with Pro-Oxidant compounds. Data points indicate the mean average of 6 replicate wells, with error bars indicating the Standard Error of the Mean. Graphs and statistics were generated using GraphPad Prism.

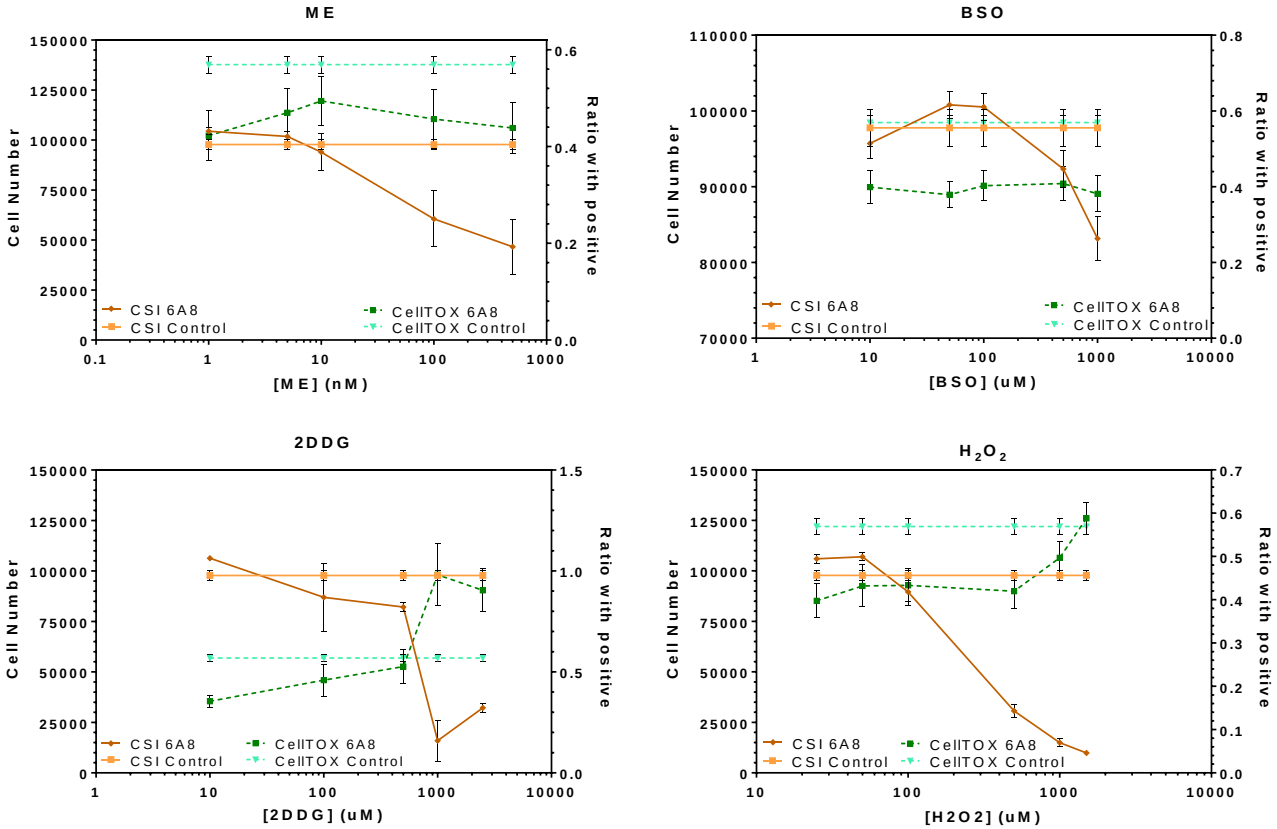

Figure AB.2. Dose Response curves for producer cell line 6A8 when treated with Pro-Oxidant compounds. Data points indicate the mean average of 6 replicate wells, with error bars indicating the Standard Error of the Mean. Graphs and statistics were generated using GraphPad Prism.

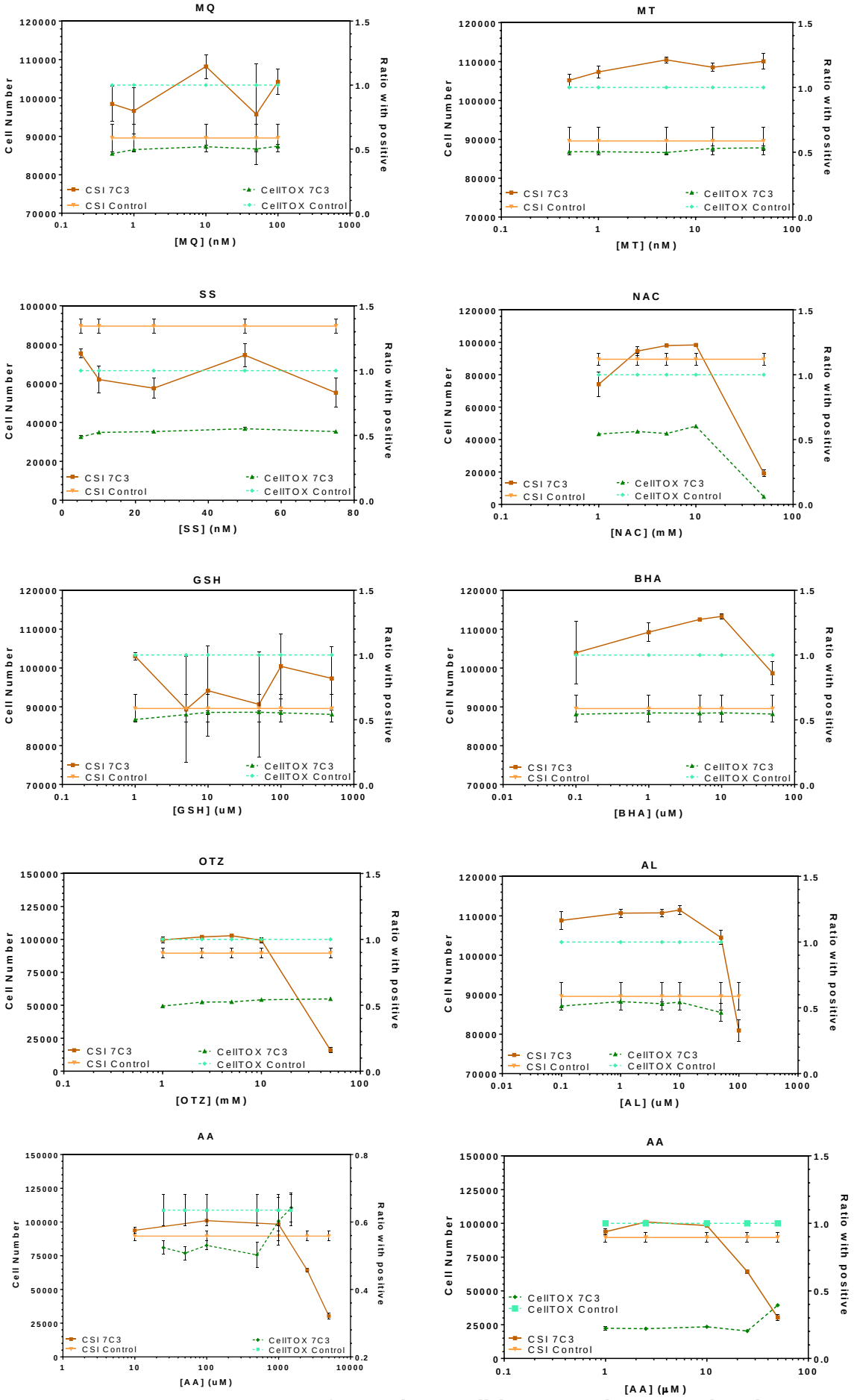

Figure AB.3. Dose Response curves for producer cell line 7C3 when treated with Anti-Oxidant compounds. Data points indicate the mean average of 6 replicate wells, with error bars indicating the Standard Error of the Mean. Graphs and statistics were generated using GraphPad Prism.

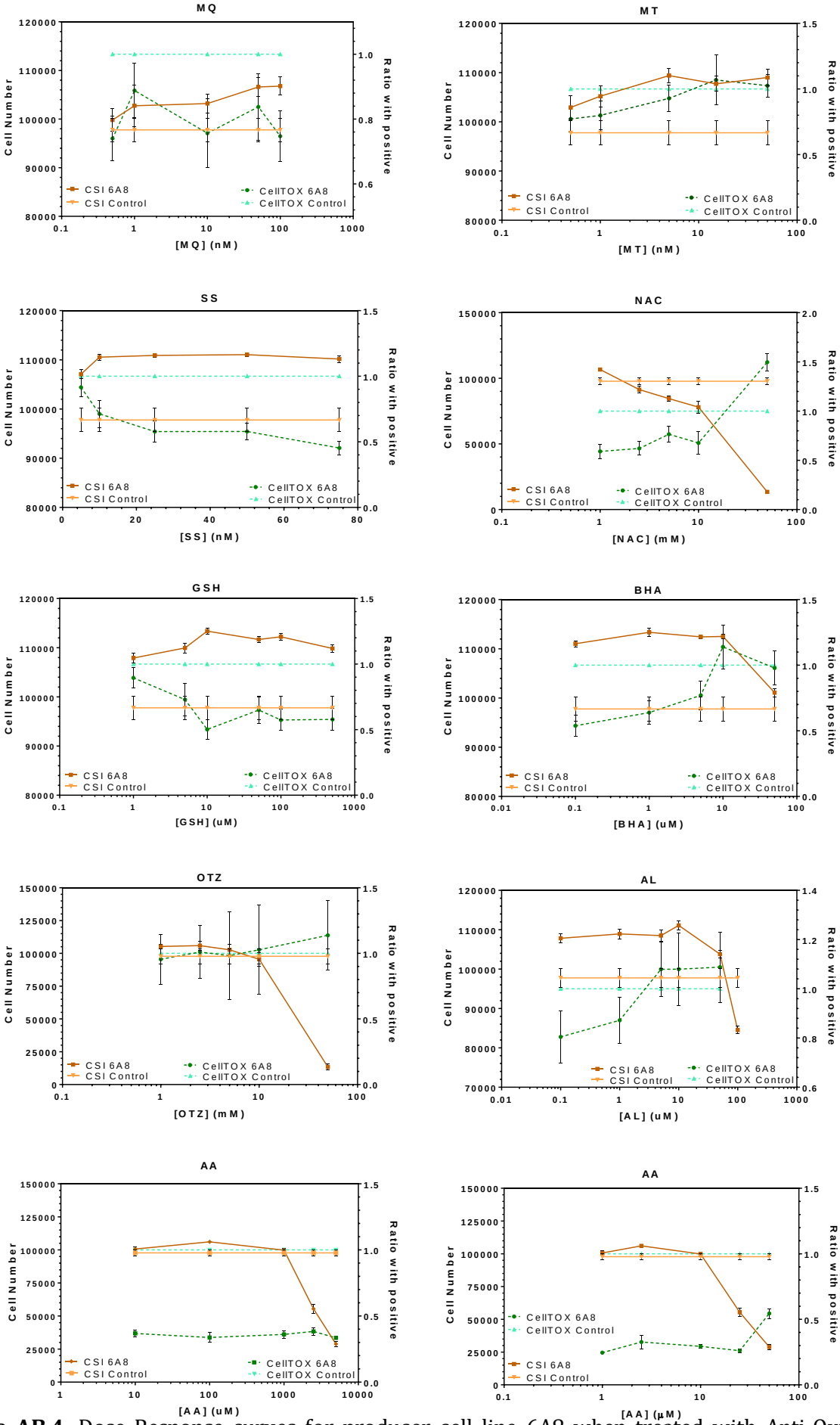

Figure AB.4. Dose Response curves for producer cell line 6A8 when treated with Anti-Oxidant compounds. Data points indicate the mean average of 6 replicate wells, with error bars indicating the Standard Error of the Mean. Graphs and statistics were generated using GraphPad Prism.

Appendix C: Design of Experiments Histograms

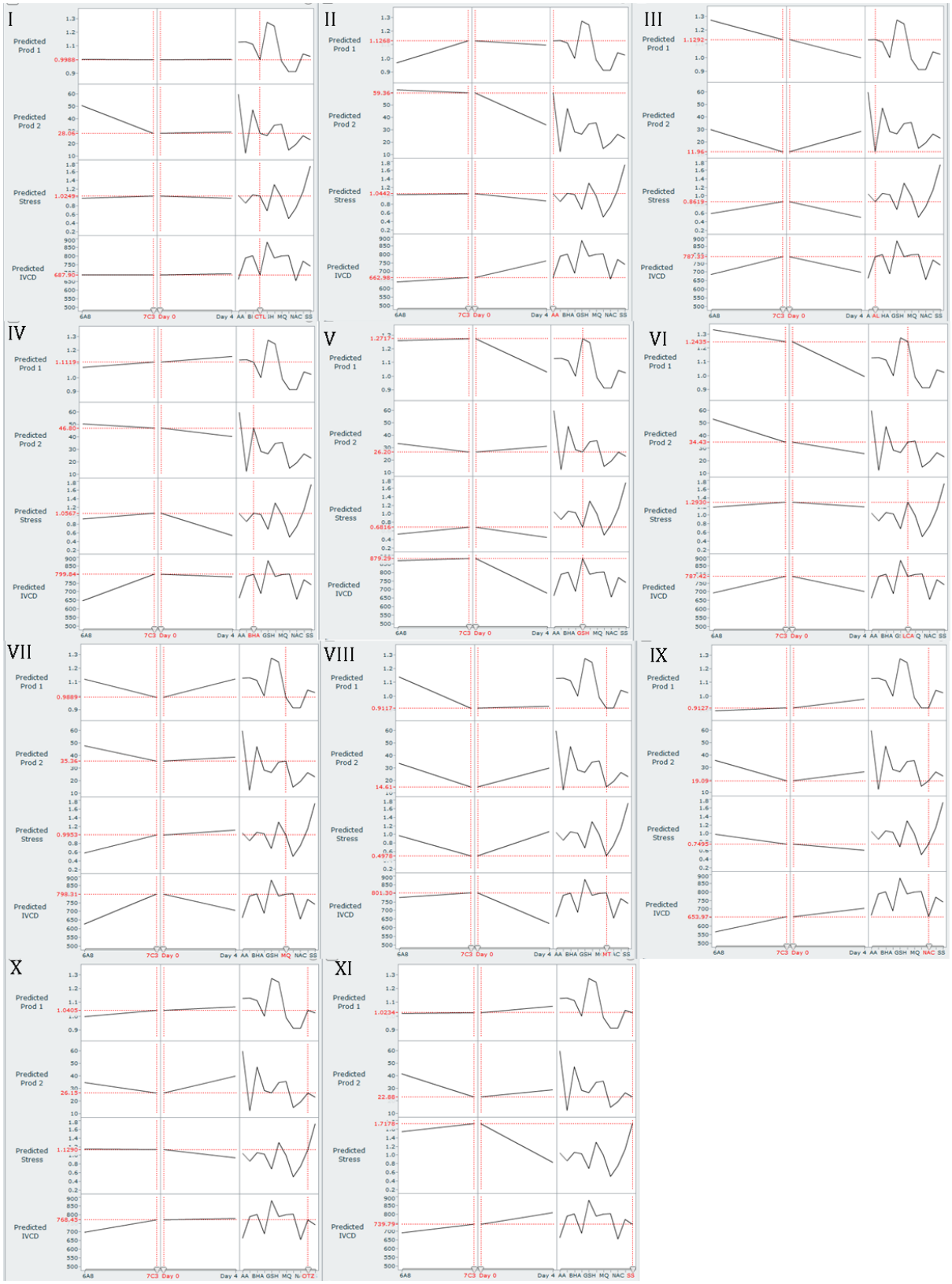

Figure AC.1. DoE Histograms of predicted productivity, growth and reactive oxygen species levels in 7C3 cell lines in a batch process treated with anti-oxidants on Day0 of culture. I. Untreated Cultures. II. Ascorbic Acid. III. α-Lipoic Acid. IV. Butylated Hydroxyanisole. V. L-Carnosine. VI. Glutathione. VII. MitoQ. VIII. MitoTEMPOL. IX. N-Acetylcysteine. X. Procysteine. XI. Sodium Selenite

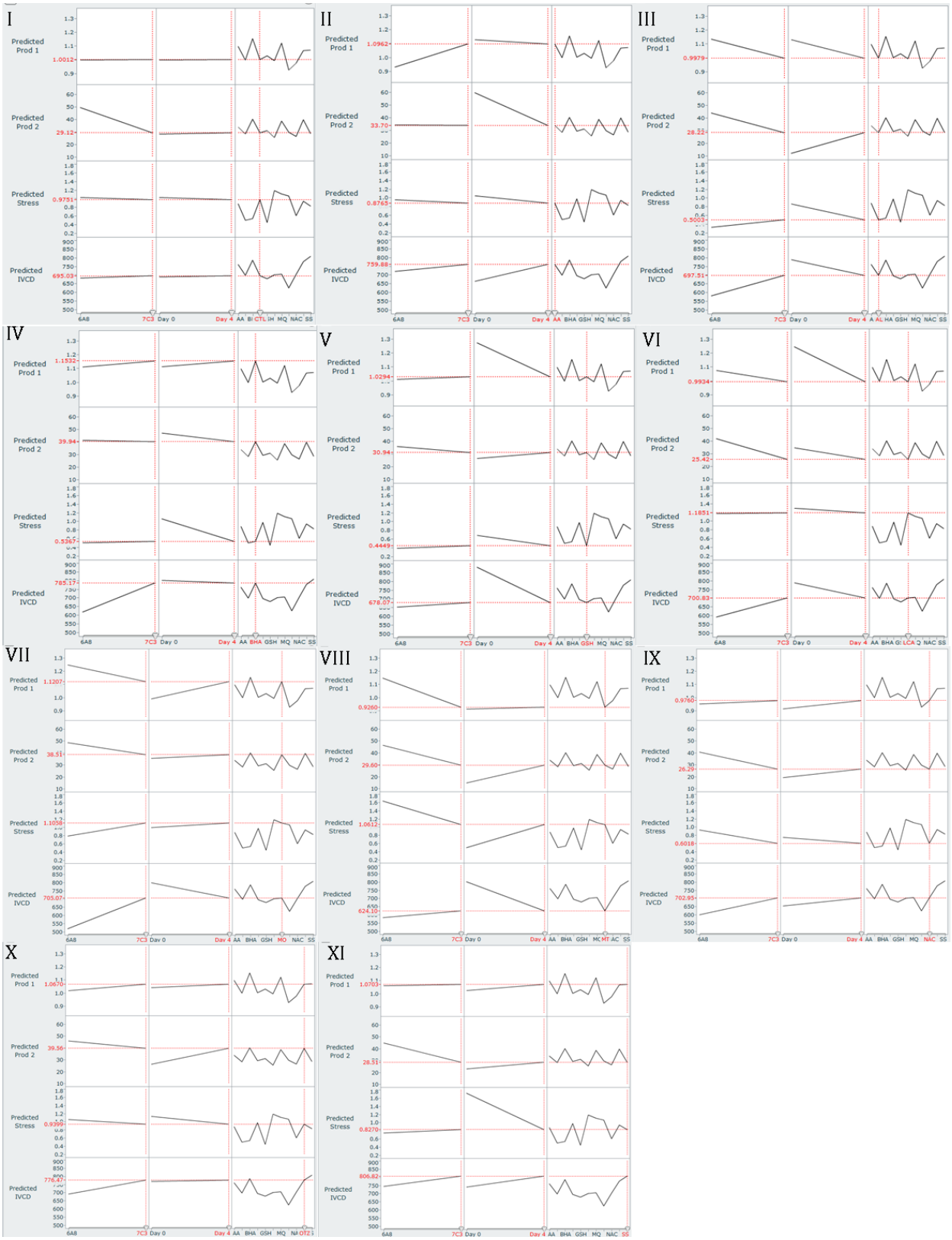

Figure AC.2. DoE Histograms of predicted productivity, growth and reactive oxygen species levels in 7C3 cell lines in a batch process treated with anti-oxidants on Day4 of culture. I. Untreated Cultures. II. Ascorbic Acid. III. α-Lipoic Acid. IV. Butylated Hydroxyanisole. V. L-Carnosine. VI. Glutathione. VII. MitoQ. VIII. MitoTEMPOL. IX. N-Acetylcysteine. X. Procysteine. XI. Sodium Selenite

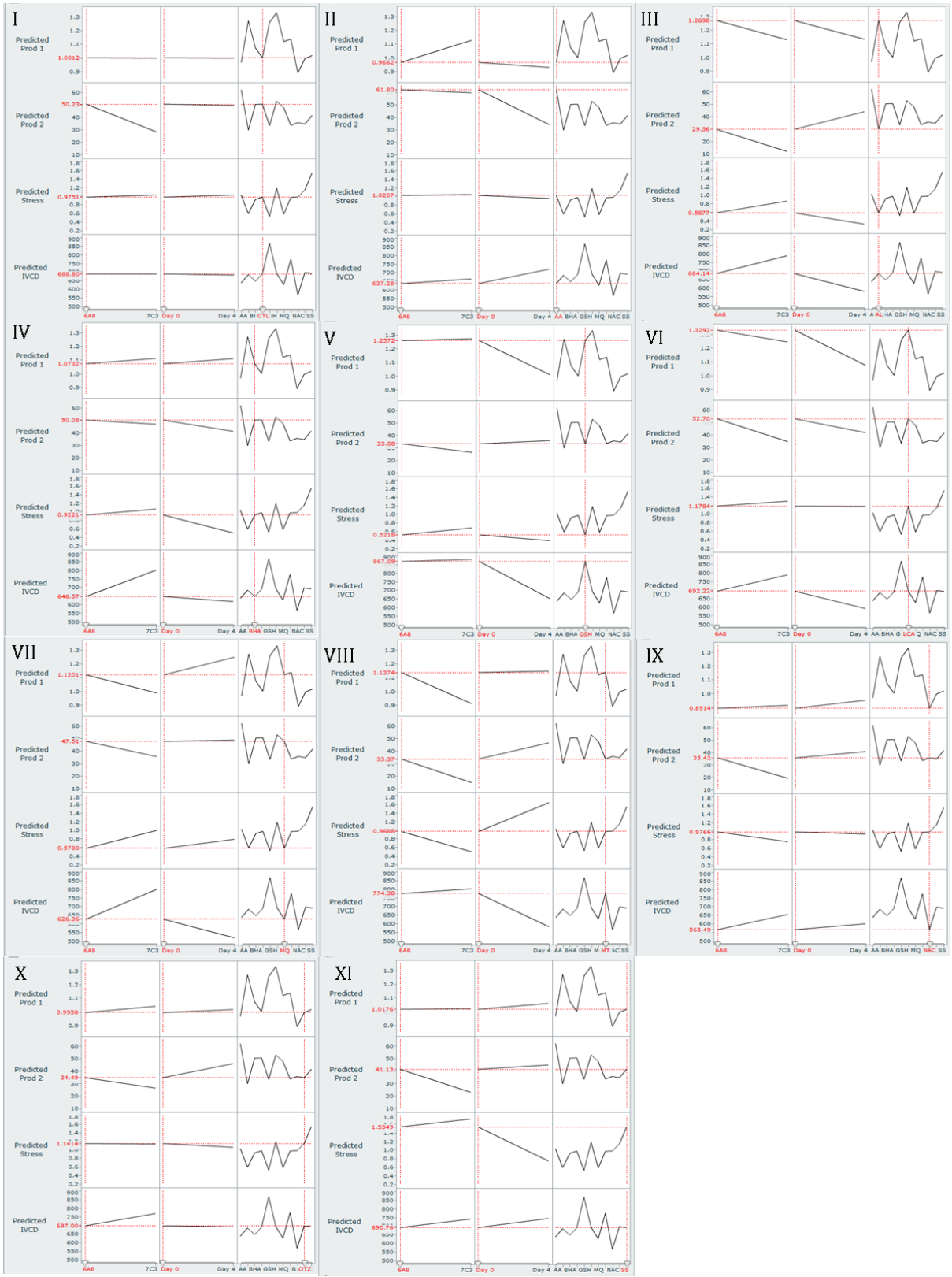

Figure AC.3. DoE Histograms of predicted productivity, growth and reactive oxygen species levels in 6A8 cell lines in a batch process treated with anti-oxidants on Day0 of culture. I. Untreated Cultures. II. Ascorbic Acid. III. α-Lipoic Acid. IV. Butylated Hydroxyanisole. V. L-Carnosine. VI. Glutathione. VII. MitoQ. VIII. MitoTEMPOL. IX. N-Acetylcysteine. X. Procysteine. XI. Sodium Selenite

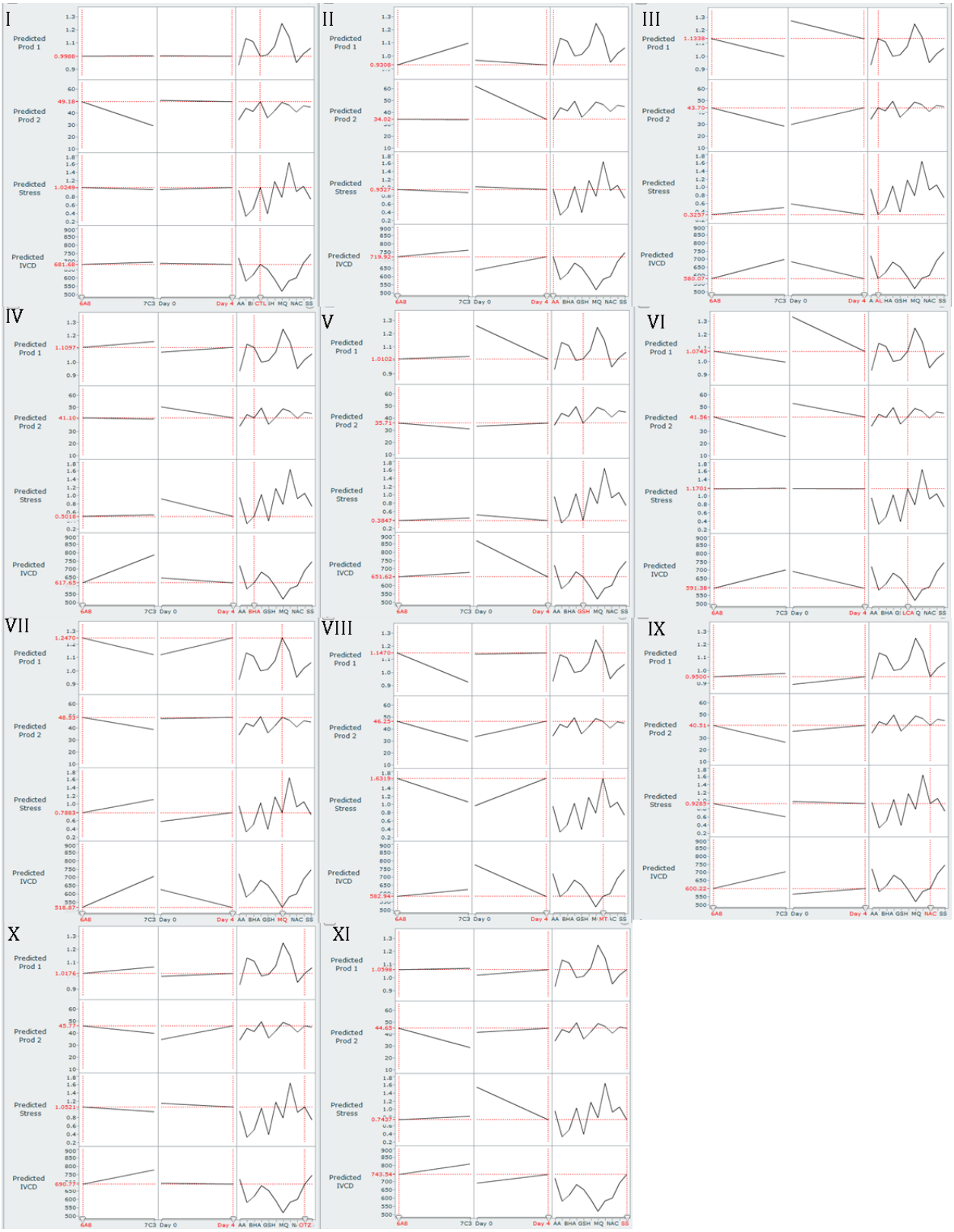

Figure AC.4. DoE Histograms of predicted productivity, growth and reactive oxygen species levels in 6A8 cell lines in a batch process treated with anti-oxidants on Day4 of culture. I. Untreated Cultures. II. Ascorbic Acid. III. α-Lipoic Acid. IV. Butylated Hydroxyanisole. V. L-Carnosine. VI. Glutathione. VII. MitoQ. VIII. MitoTEMPOL. IX. N-Acetylcysteine. X. Procysteine. XI. Sodium Selenite

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