Intracellular Response to Process Optimization and Impact on Productivity and Product Aggregates for a high-titer CHO Cell Process †

Running Title: Intracellular Response to Process Changes

Michael W. Handlogten*¹, Allison Lee-O’Brien¹*, Gargi Roy², Sophia V. Levitskaya³, Raghavan Venkat¹, Shailendra Singh⁴#, and Sanjeev Ahuja¹#

*Indicates Equal Contribution

¹Cell Culture & Fermentation Sciences, MedImmune LLC, 1 Medimmune Way, Gaithersburg, MD 20878
²Antibody Discovery & Protein Engineering, MedImmune LLC, 1 Medimmune Way, Gaithersburg, MD 20878
³Analytical Sciences, MedImmune LLC, 1 Medimmune Way, Gaithersburg, MD 20878
⁴Bio Pharmaceutical Development & Commercialization, 770 Sumneytown Pike, West Point, PA 19486


†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/bit.26460]
Abstract

A key goal in process development for antibodies is to increase productivity while maintaining or improving product quality. During process development of an antibody, titers were increased from 4 to 10 g/L while simultaneously decreasing aggregates. Process development involved optimization of media and feed formulations, feed strategy, and process parameters including pH and temperature. To better understand how CHO cells respond to process changes, the changes were implemented in a stepwise manner. The first change was an optimization of the feed formulation, the second was an optimization of the medium, and the third was an optimization of process parameters. Multiple process outputs were evaluated including cell growth, osmolality, lactate production, ammonium concentrations, antibody production, and aggregate levels. Additionally, detailed assessment of oxygen uptake, nutrient and amino acid consumption, extracellular and intracellular redox environment, oxidative stress, activation of the unfolded protein response (UPR) pathway, protein disulfide isomerase (PDI) expression and heavy and light chain mRNA expression provided in-depth understanding of the cellular response to the process changes. The results demonstrate that mRNA expression and UPR activation were unaffected by process changes, and that increased PDI expression and optimized nutrient supplementation are required for higher productivity processes. Furthermore, our findings demonstrate the role of extra- and intracellular redox environment on productivity and antibody aggregation.Processes using the optimized medium, with increased concentrations of redox modifying agents, had the highest overall specific productivity, reduced aggregate levels, and helped cells better withstand the high levels of oxidative stress associated with increased productivity. Specific productivities of different processes positively correlated to average intracellular values of total glutathione. Additionally, processes with the optimized media maintained an oxidizing intracellular environment that is important for correct disulfide bond pairing, which likely contributed to reduced aggregate formation. These findings shed important understanding into how cells respond to process changes and can be useful to guide future development efforts to enhance productivity and improve product quality. This article is protected by copyright. All rights reserved

Key Words CHO cell Culture, Nutrient Metabolism, Improved Product Quality, Redox Environment, Oxidative Stress
Introduction

Protein therapeutics have been successfully designed to treat a wide range of diseases and conditions (Weiner, 2015). The success of these treatment options has made biologics an increasingly important and growing class of therapeutics in the clinic. There are currently 52 mAbs in Phase 3 studies, which is a 100% increase from 2010 (Reichert, 2017). In order to ensure sufficient supplies of these important molecules and reduce manufacturing costs, a key goal of cell culture process development is to increase productivity, while maintaining or improving product quality (Li et al., 2010). To achieve this goal, two approaches are often applied: 1) identification of a highly productive cell line and 2) optimization of the production process for a given cell line (Li et al., 2010; Rouiller et al., 2015).

Substantial work has been conducted to correlate high producing cell lines with intracellular processes that can affect protein production. The relevant methods include measuring the expression levels of heavy chain (HC) and light chain (LC) mRNA, activation of the unfolded protein response (UPR), activation of the endoplasmic reticulum associated protein degradation (ERAD), and an analysis of the metabolic activities related to the TCA cycle (Davis et al., 2000; Dorai et al., 2006; Gilbert et al., 2013; Nishimiya, 2014; Schlatter et al., 2005). Increased HC and LC mRNA expression, the activation of the UPR or ERAD system, and increased metabolic activity have all shown some correlation with specific productivity (Dorai et al., 2006; Gilbert et al., 2013; Nishimiya, 2014). These types of measurements are often made during clone selection in order to identify the cell lines with the greatest potential for high protein production.

Typically, once a clone has been selected, process optimization work centers around increasing titer and maintaining or improving product quality through optimization of feed composition and
strategy, medium formulation, and process set-points, such as temperature and pH (Li et al., 2010). The effects of process changes are usually determined by analyzing process data including the viable cell density (VCD), cell viability, osmolality, lactate and ammonia production, nutrient consumption, protein production, and product quality. A commonality among these measurements is that these parameters are measured largely from cell culture supernatant, despite the fact that protein synthesis occurs solely inside the cell. It is not common that intracellular responses to process changes are analyzed (Gomez et al., 2012; Sou et al., 2015). To the authors’ knowledge, such a detailed study looking into various intracellular processes that may affect productivity and product aggregates in a high-titer CHO process has not been reported.

In this work, to gain an in-depth understanding of the cellular response to process changes, we analyzed both the standard process data and the intracellular response for four different processes (ranging from ~4 to 10 g/L titer), all using the same Chinese hamster ovary (CHO) cell line. Each process was designed as a stepwise change to the previous one and the four processes resulted in different growth, productivity, and aggregate levels. This enabled us to directly determine how each process change affected the intracellular environment and how this in turn affected cell growth, productivity, and product quality. The stepwise changes were: optimization of the feed, optimization of the medium, and finally, optimization of process parameters and feed strategy. Our results demonstrate the importance of optimized nutrient feed and PDI expression for increased specific productivity and notably, the significant role of the extra- and intracellular redox environment to further increase specific productivity and decrease aggregates. These findings may help other researchers to develop cell culture processes that deliver high productivity and superior product quality.

This article is protected by copyright. All rights reserved
Materials and Methods

Antibody Production & Measurement of Extracellular Redox Potential

For all the studies described in this work, a stably transfected CHO-GS cell line that produced a mAb was used. The fed-batch processes were run in 3 L glass stirred tank bioreactors using proprietary media and nutrient feeds with an initial working volume of 1.5 L as previously described (Handlogten et al., 2017). Temperature, pH, dissolved oxygen, and agitation were monitored and controlled using SLC 5000 controller software (Rockwell Automation; Arlington, VA). Extracellular redox was monitored using an ORP probe (Mettler, Columbus, OH).

Rapidpoint® 400 Blood Gas Analyzer (Siemens; Malvern, PA) and BioProfile 400 (NOVA Biomedical; Waltham, MA) were used to measure offline pH, gasses (pCO₂, pO₂), and metabolites (glucose, lactate, ammonium). Cell density and viability were measured using a Vi-CELL XR (Beckman Coulter; Brea, CA). Osmolality was measured using an osmometer (Advanced Instruments, Inc; Norwood, MA). Titer was measured on a 4.6 x 50 mm Protein A column (Thermo Fisher Scientific, Waltham, MA) using an HPLC (Agilent Technologies; Santa Clara, CA).

Measurement of Reactive Oxygen Species by Flow Cytometry

Reactive oxygen species were measured to assess the intracellular redox of the cell based on a previously published protocol (Cossarizza et al., 2009). Cells were stained with 4 µM of 2’, 7’-dichlorodihydrofluorescein diacetate (H₂DCF-DA) (Thermo Fisher Scientific) to detect H₂O₂, and 25 nM TO-PRO-3 (Invitrogen; Carlsbad, CA) to detect viability. 20µM Menadione (MEN, Alfa Aesar; Ward Hill, MA) was used as a positive control. Stained samples were run on an LSR II flow cytometer (BD; Franklin Lakes, NJ).

This article is protected by copyright. All rights reserved
Measurement of Glutathione

Cells culture samples were quenched in a methanol and ammonium bicarbonate buffer and metabolites were extracted in methanol using a previously described procedure (Sellick et al., 2011). Extracted glutathione was detected using a GSH/GSSG (reduced to oxidized glutathione) Ratio Detection Assay Kit (Abcam; Cambridge, UK) following the manufacturer’s protocol. The data generated from this protocol was also used to estimate the total amount of glutathione present inside the cells.

Amino Acid Analysis

Cell culture supernatants were filtered through 10 kDa filters and filtrates were diluted using HPLC grade water to appropriate concentrations. Amino Acid Standard (Waters; Milford, MA) was diluted for use as a standard curve. Diluted samples and standards were combined with internal standard Norvaline (Sigma-Aldrich) and AccQ Tag Derivation Kit reagents (Waters). Samples were run on an Acquity UPLC system (Waters) using an AccQ-Tag Ultra RP Column 130Å 1.7 µm, 2.1 mm 100 mm (Waters). Data was analyzed using EMPOWER software (Waters).

RNA Isolation and Quantitative PCR (qPCR)

Total RNA was isolated from cell pellets using an RNeasy plus mini kit (Qiagen), and reverse transcription (RT) was performed using TaqMan Reverse Transcription Reagents (Life Technologies, Grand Island, NY, USA) with 1.0 µg RNA according to the manufacturer’s protocol. LC, HC, and GAPDH mRNA expression levels were measured using TaqMan technology on the 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). The probes and primers were generated by assays-by-design (Applied Biosystems); the probes contained a 6-
carboxy-fluoresceinphosphoramidite (FAM dye) label at the 5’ end of the oligo and a nonfluorescent quencher at the 3’ end. qPCR was carried out by denaturing at 95°C for 20 seconds and then cycling at 95°C for 1 second and 60°C for 20 seconds, for 40 cycles. Chinese hamster specific GAPDH was used as the endogenous RNA control. Expression levels of LC and HC were normalized to endogenous CHO GAPDH.

**Western Blot Analysis**

Cells were lysed in M-PER, mammalian protein extraction reagent (ThermoFisher Scientific) containing Halt Protease (ThermoFisher Scientific) following manufacturer’s recommendations. Twenty micrograms of cell free extracts were analyzed on SDS-PAGE (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membrane using Trans-Blot Turbo transfer system (Bio-Rad). Membranes were probed with rabbit anti-PDI, (Cell Signaling, Boston, MA, USA) and chicken anti-α-Tubulin reactive to human species (Abcam). α-Tubulin was used as a loading control. Antigen specific bands were detected by incubation of membranes using HRP-conjugated anti-rabbit or anti-mouse antibodies followed by incubation with SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific) and image analysis by ImageQuant LAS4000 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). HC and LC were detected using HRP-conjugated anti-human Fc specific (Jackson Immuno Research labs, West Grove, PA, USA) and anti-human kappa light chain specific (Bethyl Laboratories Inc., UK) antibodies.

**Aggregation Level Determination**

Total aggregates in cell-free samples were evaluated by High Performance Size Exclusion Chromatography (HPSEC) on a TSKgel G3000SW_{XL} column, 5 μm, 250 Å, 7.8 mm I.D. x 300 mm (Tosoh Bioscience, Montgomeryville, PA), using an Agilent 1200 HPLC system (Agilent...
Technologies, Santa Clara, CA) operated by Agilent ChemStation software. Protein elution was monitored by UV absorbance at 280 nm.

**Results and Discussion**

**Experimental Design**

To improve understanding of the cellular response to process changes, a CHO cell line producing a monoclonal antibody was run under four different conditions as outlined in Table 1 and samples from these processes were analyzed for changes in growth, productivity, mRNA expression, UPR activation, PDI expression, metabolites, and oxidative stress. These process conditions were selected based on their diverse cell growth, productivity, and product quality profiles outlined in Table 2. By introducing minimal changes between the processes, we were able to determine the contribution of each process change on the intracellular response and consequently, better understand how and why each process change affects the cell growth, productivity, and product quality.

**Process Data**

The four processes outlined in Table 1 were run in duplicate 3L stirred tank bioreactors using the same CHO cell line. The conditions in Process A resulted in moderate cell growth, maintained cell viability >70% throughout the run duration, had relatively low ammonium concentrations and a favorable lactate profile (Figure 1A, B, G, H). Lactate, which is a byproduct of anaerobic glycolysis, was initially produced until day 4 after which it was consumed and remained at undetectable levels until day 14. However, Process A had relatively low titer, only reaching 4.2 g/L, and resulted in product comprising 22% total aggregates (Figure 1C, I). Process B, using the enriched nutrient feed, maintained similar cell viabilities and resulted in increased peak VCD,
increased specific productivity, and greater than twice the titer of Process A (Figure 1A, B, C, E). However, the product still had high levels of aggregates (21%), increased levels of ammonium, and a large lactate spike towards the end of the run (Figure 1G, H, I). A new basal medium was developed for Process C with increased levels of some components including redox modifying agents. The new medium resulted in approximately 50% reduction in peak VCD, 15% reduction in titer, increased osmolality, similar ammonium concentration and a steeper end of run lactate spike compared to Process B (Figure 1A, C, D, G, H). Beneficially, the specific productivity was significantly increased (300% compared to Process A) and the levels of aggregates were reduced by 20% (Figure 1E, I). Previous work has shown that oxidizing conditions and increased osmolality can limit cell growth; it is likely that increases in both of these parameters (as evidenced by extracellular osmolality and culture redox potential for majority of the process duration) compared to processes A and B contributed to the reduction in peak VCD (Figure 1D,F) (Gille and Joenje, 1992; Zhu et al., 2005). Conceivably, these two parameters interacted to restrict cell growth. The higher initial cell culture redox potential may have restricted cell growth in the exponential phase of the culture resulting in nutrient accumulation, which then led to increased osmolality and a further reduction in peak VCD.

The final process, with optimized feed strategy, pH, and temperature resulted in reduced osmolality, ammonium, and end of run lactate compared to Process C (Figure 1D, G, H). These changes in Process D moderately restored peak VCD and increased titers to 10 g/L while maintaining the high specific productivity and reduced aggregates. Process D, with the more oxidizing medium, had similar osmolality compared to Processes A and B (that had lower oxidizing medium) and yet had reduced peak VCD. These results provided further evidence that increased cell culture redox potential limits cell growth.
Despite using the same cell line, there were significant differences in process data and aggregate levels between processes. It is widely known that high concentrations of ammonium, lactate, and osmolality can be detrimental to cell culture performance (Slivac et al., 2010; Zhu et al., 2005). However, these effects on their own could not explain the differences observed in our data. For example, Process C had the highest overall osmolality, ammonia, and end of run lactate yet had significantly higher specific productivity and end of run viability compared to Processes A and B. Thus, in an effort to better understand the differences observed in Figure 1, we examined changes in the intracellular responses between the four processes.

**HC and LC mRNA Expression**

The first step in antibody expression is the transcription of mRNA coding for the HC and LC of the antibody. Poor expression of either HC or LC mRNA can result in reduced titer (Schlatter et al., 2005). Thus, the first aspect we looked at in understanding the intracellular response to different process conditions was an analysis of mRNA expression for the HC and LC. Cell pellets were taken on days 0 and 10 from each condition and the expression of the HC and LC were evaluated using qPCR. The results in Figure 2 demonstrate that all conditions except condition B had similar expression levels of the HC and LC on both days 0 and 10. Additionally, the differences in mRNA levels did not correlate with differences in productivity. These results demonstrate that mRNA transcription was minimally impacted by process changes and likely did not contribute to the differences in VCD, specific productivity, titer or levels of aggregates. Furthermore, these results confirm previous reports that mRNA expression is a poor predictor of protein expression (Vogel and Marcotte, 2012).

**Translation, UPR Activation, and PDI Expression**

This article is protected by copyright. All rights reserved
Next, we analyzed expression of the antibody HC and LC as well as the expression levels of markers known to affect protein production and folding. We first analyzed the intracellular expression levels of the HC and LC by western blots run under reducing and non-reducing conditions (Figure 3A). The reducing conditions demonstrated that both the HC and LC were well expressed and that Processes B, C, and D had increased expression levels relative to Process A. The non-reducing conditions illustrated that the antibody was well assembled and secreted on the whole as there was no significant accumulation of antibody or incomplete antibody (e.g., missing a light chain) during the processes. A similar analysis of the supernatant confirmed the secretion of intact antibody without the detection of antibody fragments (Figure 3B). However, the western blots shown in Figure 3 provide only a static view of antibody expression at the time the samples were taken and do not provide insight into factors affecting the rates at which the antibody is being expressed.

We therefore evaluated several markers of the UPR as well as expression of PDI. Protein folding and post-translational modifications including glycosylation and disulfide bond formation are processed in the ER. Improperly folded proteins are tagged for degradation via endoplasmic reticulum-associated degradation (ERAD) or sent back through the folding cycle (Ruggiano et al., 2014). An accumulation of improperly folded proteins activates the UPR pathway (Chakrabarti et al., 2011). Activation of the UPR pathway induces the expression of chaperones that enhance protein folding that in turn can reduce the amount of unfolded protein and increase antibody production. UPR can also limit translation in an effort to reduce the amount of unfolded protein and can cause apoptosis in response to sustained high levels of unfolded protein (Hetz, 2012). To assess the activation of the UPR and ERAD, we ran western blots for spliced and un-spliced x-box binding protein 1 (XBP-1), binding immunoglobulin protein (BiP), phosphorylated
proteins, the protein kinase RNA-like ER kinase (p-PERK), and the protein disulfide isomerase (PDI). Spliced XBP-1 is an indicator of induction of UPR (Hetz, 2012). Similarly, BiP is a chaperone that helps with protein folding and its expression is increased when the UPR is active (Hetz, 2012). The phosphorylation of PERK is an indicator of the protein expression inhibitory pathway associated with the UPR pathway (Hetz, 2012). PDI can be upregulated by the UPR and is an important enzyme that contributes to disulfide bond formation during protein folding (Hatahet and Ruddock, 2009). PDI can also facilitate refolding of proteins with incorrectly paired disulfide bonds and in addition is part of the ERAD pathway and helps in the degradation of terminally misfolded proteins (Grubb et al., 2012).

The analysis for each of these proteins was run on cell pellets from days 0, 6, 8, 10, 12, and 14. All samples showed similar ratios of spliced to un-spliced XBP-1, high expression levels of BiP, and undetectable levels of p-PERK (data not shown). Combined, these results demonstrate a favorable activation of the UPR system for all four processes; the expression of chaperones that facilitate protein folding were upregulated without activation of the expression inhibitory pathway. Finally, we evaluated the expression of PDI using \( \alpha \)-tubulin as a loading control (Figure 4A). The expression levels of \( \alpha \)-tubulin and other commonly used loading control proteins (not shown) decreased towards the end of the run for all four processes despite equal protein loading. The reduced expression of these loading control proteins is due to reduced viability and has been observed in-house for multiple CHO cell processes. Our results show that PDI expression relative to \( \alpha \)-tubulin increased during the run for all four processes and that the increase in PDI expression was greater for Processes B, C, and D (Figure 4B). Due to the important role of PDI in antibody disulfide bond formation, it is likely that the increased expression of PDI supported the increased specific productivity in Processes B, C, and D. It
however is unclear whether greater PDI expression leads to increased specific productivity or if increased specific productivity combined with UPR activation resulted in increased PDI expression. Additionally, PDI expression alone does not explain the variations observed in cell growth or aggregate levels; however, the results do demonstrate that PDI plays an important role in high productivity processes.

**Metabolic Analysis**

We analyzed specific amino acid consumption rates to evaluate differences in metabolism between the four processes. The concentration of each amino acid in the cell culture supernatant was determined on each day of the run and the daily specific consumption rate of each amino acid was calculated. The specific amino acid consumption rates during the mid-run phase of the culture (days 6-11) are shown in Figure 5. In addition, the amino acid analysis revealed that Process A ran out of several amino acids including glycine, alanine, cystine, methionine, aspartic acid, asparagine, serine, and proline. Some of these amino acids were depleted during the exponential phase of the process. Considering the timing and number of amino acids that were depleted during the run, insufficient nutrients were the most likely cause of low specific productivity in Process A as compared to other processes. Process B, with the enriched nutrient feed, did not have the same nutrient limitations as in Process A and resulted in increased specific consumption rates of nearly every amino acid. The enriched nutrient feed in Process B supported higher cell growth and specific productivity compared to Process A. The increase in consumption rates and corresponding increase in productivity highlights the importance of optimized nutrient levels for highly productive processes. Process C had similar specific amino acid consumption rates compared to Process B with the exception of alanine and cystine. Process C had a net production of alanine and increased consumption of cystine. Both Process B and C
used the same nutrient feed strategy. However, approximately 50% reduction in peak VCD between these processes contributed to a buildup of nutrients and subsequently increased osmolality during the run in Process C. Increased osmolality has been shown to reduce cell growth (Zhu et al., 2005). Additionally, excess amino acids are not stored by cells but are instead metabolized releasing a stoichiometric amount of ammonia (Chen and Harcum, 2005). The excess nutrient feed likely contributed to the elevated levels of ammonia observed for this process (Figure 1H). Process D, with the optimized feed strategy, had the highest overall specific consumption rates for nearly all amino acids. Process D still ran out of three amino acids, however, this was not until the end of the process. Additionally, there was no significant drop in specific productivity at the end of this process so it is unlikely that supplementation of these amino acids would have further improved the process. Overall, the minor changes in Process D that reduced nutrient build up, osmolality, and ammonia created a more favorable environment for cell growth while maintaining high productivity.

To further understand the differences in metabolism between processes, we calculated the specific glucose, lactate, and oxygen uptake rates (OUR) (Figure 6). The specific OUR was calculated using the gas flow rate, volumetric mass transfer coefficient ($k_L a$) of the vessel, and culture VCD. Cells metabolize glucose to pyruvate, which intersects several metabolic pathways. An important use of pyruvate is the generation of ATP through either oxidative metabolism using the TCA cycle or fermentative metabolism that results in lactate production (Martínez et al., 2013). All four processes had similar glucose and lactate consumption rates during the growth phase. The glucose consumption rate decreased significantly for all four processes as the cells switched from lactate production to lactate consumption. Lactate can be metabolized back to pyruvate which reduces the requirement for glucose. Processes A, B, and D maintained
reduced glucose consumption rates until the end of the run while Process C glucose consumption rate increased toward the end. The cells in Process C switched back to lactate production converting the additional glucose to lactate as indicated by the significant end of run lactate production (Figure 6A, B). OUR is reflective of oxidative phosphorylation, which is a more efficient method of ATP generation compared to fermentative metabolism (Luo et al., 2012). Therefore, increased OUR with similar glucose consumption indicates more efficient glucose usage. Processes C and D had increased OURs over the duration of the run (Figure 6C). It is likely that the increased OURs observed in Processes C and D partly facilitated the increased specific productivities.

Ammonia, a byproduct of amino acid catabolism, provides additional insight into cell metabolism. All four processes had the same initial rate of ammonia production followed by either a decline or leveling off of ammonia levels mid-culture and finally an increase in ammonium levels towards the end of the run (Figure 1H). The initial increase in ammonium levels was due to the initial high specific consumption rates of amino acids during the growth phase (data not shown). The decrease or leveling off of ammonium levels in the middle of the run coincided with the accumulation of glutamine and increased glutamate consumption (data not shown). This cell line was designed using the GS system that facilitates the production of glutamine via the reaction of ammonia and glutamate using the enzyme glutamine synthetase (Fan et al., 2012). The concentrations of ammonium decreased until lactate was depleted or in the case of Process C, remained unchanged, until the cells began producing lactate again. This has been observed previously and has been attributed to the conversion of alanine to pyruvate to maintain carbon flux into the TCA cycle (Li et al., 2012). The conversion of alanine to pyruvate can proceed using either alanine dehydrogenase or alanine transaminase and glutamate
dehydrogenase; both processes produce ammonia at a stoichiometric rate. Our data supports this mechanism; the onset of lactate depletion, initiation of alanine consumption, and production of ammonium all happened simultaneously. Additionally, the molar concentration decrease in alanine corresponded to the molar increase in ammonium concentration. It is important to note that Process C never fully consumed lactate and as expected did not consume alanine. Processes B and C also had an end of run lactate spike. The spike in lactate coincided with the end of glutamine production, which might have further contributed to the high levels of ammonium in Processes B and C. The lactate spike towards the end of the process can indicate reduced mitochondrial oxidative capacity (Gilbert et al., 2013; Zhang et al., 2015). Oxygen is consumed primarily in the mitochondria for ATP synthesis and oxygen uptake is an indicator of mitochondrial function (Wagner et al., 2011). An analysis of the specific OUR revealed that Processes B and C had a decrease in specific OUR on days 13 and 14 relative to Processes A and D (Figure 6C). These differences might be related to nutrient limitations, process conditions, or mitochondrial function and need further scrutiny.

Summarizing the metabolic analysis, during the growth phase, all four processes had high specific consumption rates for amino acids, high glucose consumption rates, and high lactate production rates. Mid-run, lactate was consumed replacing some of the glucose as a source for pyruvate. At the same time, the cells began producing glutamine from glutamate thereby limiting and even decreasing ammonium concentrations. The cells’ metabolic activities continued in this manner until lactate was fully consumed at which time the cells began converting alanine to pyruvate, a process that created a stoichiometric increase in ammonium. In the event of an end of run lactate spike, as in Processes B and C, the metabolic activity again changed as the OUR decreased, glucose consumption increased, and glutamine production ceased. The combination
of glucose, lactate, ammonium, amino acids, and OUR enabled an in-depth analysis of the metabolic activity of the cell line when run under four different processes and emphasizes the importance for an appropriate nutrient feed. Insufficient nutrient feed as in Process A led to reduced productivity while over feeding as in Process C led to increased ammonium and osmolality and an end of run lactate spike. This analysis even suggested a potential improvement of Process D as discussed below. High concentrations of ammonia are widely known to adversely affect cell culture (Slivac et al., 2010). The spike in ammonium concentration in the second half of the run could potentially be mitigated by supplementing the culture with pyruvate or lactate to negate the requirement of the conversion of alanine to pyruvate (Li et al., 2012).

**Intracellular Redox**

Recent studies have demonstrated that the intracellular redox environment plays an important role in the specific productivity of a cell line (Chong et al., 2012; Templeton et al., 2013). Consequently, we evaluated the intracellular redox by evaluating the accumulation of reactive oxygen species and both oxidized and reduced glutathione. Reactive oxygen species (ROS) including superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH$^-$) are all produced as by-products of aerobic metabolism (Murphy, 2009). The intracellular accumulation of ROS leads to oxidative modifications of proteins and DNA strand breaks that if left unchecked eventually lead to loss of molecular function and cell death. The glutaredoxin system is a major pathway that cells use to manage oxidative stress (Lillig et al., 2008). This enzymatic system is maintained by reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). Intracellular glutathione concentrations can exceed 10 mM and the ratio of reduced to oxidized glutathione is commonly used to evaluate intracellular oxidative stress (Lillig et al., 2008). To evaluate differences in oxidative stress across the four processes, we determined the...
intracellular levels of H$_2$O$_2$ by flow cytometry and the ratio of reduced to total glutathione using metabolite extraction.

The intracellular levels of H$_2$O$_2$ remained constant for all conditions for the first 7 days, after which the H$_2$O$_2$ levels increased for all processes (Figure 7A). Process A experienced the smallest increase in H$_2$O$_2$ levels, which is likely a reflection of the overall low productivity. Process B, with significantly increased productivity, had the greatest overall increase in H$_2$O$_2$. Intriguingly, Processes C and D, which had the highest overall specific productivities, had lower end of run H$_2$O$_2$ levels compared to Process B, thereby demonstrating that productivity is not the only factor affecting intracellular accumulation of H$_2$O$_2$. In the absence of high levels of oxidative stress, cells typically maintain greater than 85% of glutathione in a reduced state (Lillig et al., 2008). Curiously, the ratio of reduced to total glutathione was between 0.2 and 0.4 for all processes up to day 10 indicating that the cells were managing substantial oxidative stress (Figure 7B). However, on day 14, Processes A and B had increased levels of reduced to total glutathione compared to processes C and D. Also, the total amount of glutathione detected intracellularly for Process A was significantly lower compared to other three processes up to day 10 while end of run intracellular glutathione levels for Processes C and D were significantly higher than those of Processes A and B (Figure 7C). Collectively, this indicated that Process A had lower capacity to manage oxidative stress than other three processes up to day 10 and cells in Processes C and D had better capacity to manage oxidative stress compared to other two processes towards the end. It has been previously observed that peak antibody production is associated with a low ratio of reduced to total glutathione (Templeton et al., 2013) and that total glutathione is correlated with specific productivity (Chong et al., 2012); our results are in line with both of these observations. The specific productivity of Process A was particularly low in
the later half of the process while the specific productivity of Process B began to decrease
towards the end of the run (Figure 1E). Processes C and D maintain higher specific productivity
throughout the run. Specific productivities of different processes positively correlated to average
intracellular values of total glutathione.

One of the differences between Processes A and B compared to C and D was the use of a
medium with increased levels of redox modifying agents. The medium used in Processes C and
D, i.e. ‘Medium 2’ did not have any new components compared to those in ‘Medium 1’ (used in
Processes A and B). The changes involved only increasing the amounts of a few components; the
most significant changes were those impacting the redox potential of the medium. The net effect
of the changes was a more oxidizing extracellular environment in Processes C and D compared
to that in Processes A and B (Figure 1F). Consistent with another investigation (Takahashi et al.,
2002), the processes using the optimized media with modified culture redox had increased
specific consumption of cystine (Figure 5). Transport of cystine across the cell membrane is
generally considered the rate limiting step in the biosynthesis of glutathione (Lu, 2009).

Combined, these results suggest that the increased concentrations of redox modifying agents
likely facilitated increased GSH synthesis by greater cystine uptake, thereby enabling the cells to
to better manage the increased oxidative stress associated with high productivity and to maintain
peak productivity through the end of the process.

**Aggregate Analysis**

To further understand the formation of aggregates, we analyzed aggregates via western blot
analysis on both cell lysates and supernatant samples (Figure 3). The results of the non-reducing
western blot of the cell lysates from days 0 and 10 revealed the presence of intermediate forms of

This article is protected by copyright. All rights reserved
the IgG. When the same samples were run under reducing conditions, primarily monomeric HC and monomeric LC were detected. Similar analysis was performed on supernatant samples from days 6, 10, and 14. The non-reducing western blot displayed primarily intact antibody. When the same samples were run under reducing conditions only monomeric HC and LC were detected. Aggregates were not quantifiable on either western blot, which is likely due to low aggregate concentrations and poor resolution in the high molecular weight range (>200 kDa) of the western blots. To investigate how different media might impact aggregate formation, purified antibody was spiked into medium 1, medium 2, cell conditioned medium 1, and cell conditioned medium 2. The antibody samples in media were incubated for 2 days at 36°C. At the end of the incubation, there was no significant increase in aggregate levels and all samples contained ~4.3% aggregates (Figure 8). Since the concentration of aggregates were not statistically different and did not increase for any of the conditions, it was concluded that neither the medium nor the secreted cellular components directly impacted aggregate levels and further that the aggregates were likely produced intracellularly.

An oxidizing environment in the endoplasmic reticulum (ER) is essential for proper formation of disulfide bonds (Hatahet and Ruddock, 2009). The redox balance of the cytosol influences the environment of the ER where antibody disulfide bonds are formed. In particular, oxidized glutathione in combination with the enzyme ER oxidoreductin (ERo1) oxidizes the disulfide bonds of PDI, which is required for PDI mediated disulfide bond formation (Hatahet and Ruddock, 2009). It is important to note that all four processes have similar levels of aggregates on days 6 and 10 and that it was not until day 14 that significant differences in aggregate levels were observed (Figure 11). Processes C and D maintained approximately the same level of aggregates, 16%, while Processes A and B increased to approximately 22%. A possible
explanation for these results lie in the ratio of reduced to total glutathione (Figure 7B). All four processes maintained a relatively low ratio of reduced to total glutathione through day 10 indicating an oxidizing intracellular environment. On day 14 however, processes A and B had a significantly higher ratio of reduced to total glutathione while processes C and D maintained nearly a constant ratio (Figure 7B). As the intracellular environment became more reducing in Processes A and B, the activity of PDI might have slowed, resulting in increased amounts of improper disulfide bond pairing during protein folding and increased aggregate formation. As we previously described, the differences in the intracellular levels of total glutathione might be due to differences in cystine uptake rates caused by the increased concentrations of redox modifying agents in medium 2. While the medium alone did not impact aggregate levels of the purified product as shown in Figure 8, the medium affected the intracellular redox environment and as a result had a significant impact on aggregate levels.

Conclusions

It is widely known that process changes can have a significant impact on productivity and product quality and yet there have been few reports on the intracellular response to such changes. Thus in an effort to understand how cells respond to process changes, we examined the product mRNA expression, UPR activation, PDI expression, metabolic profiles, and oxidative stress in addition to standard process data for a single cell line run under four different processes. Each process had a distinct growth, productivity, and product quality profile. Our results showed that mRNA expression was not significantly different across processes and therefore did not contribute to the differences in process performance and product aggregates. Similarly, UPR was active in all four processes and did not contribute to differences in culture performance and product aggregates. However, the high productivity processes all had increased expression levels
of PDI, demonstrating the importance of this enzyme. The metabolic analysis revealed the importance of feed formulation and strategy on process performance; under-feeding resulted in nutrient depletion and reduced productivity, while over-feeding led to undesirable accumulation of metabolic byproducts and high osmolality. A remarkable interplay between lactate, alanine, and ammonia was noted.

Analysis of the intracellular oxidative stress revealed the importance of redox environment and media formulation for both productivity and product aggregates. Both processes using medium 2, Process C and D, with increased redox modifying agents, had reduced ROS accumulation compared to Process B (high titer process using medium 1) despite increased specific productivity. These conditions also had average intracellular values of total glutathione and increased cystine uptake, which is often the rate limiting step for the synthesis of glutathione. Combined, these results suggest that the redox modifying agents enabled the cells to increase uptake of cystine and synthesize more glutathione. The excess glutathione enabled the cells to better manage the oxidative stress associated with high productivity. Both processes using medium 2 maintained an oxidizing environment through the end of the run and had a 20% reduction in aggregates compared to processes using medium 1. Processes using medium 1 became reducing towards the end of the run and experienced increased aggregate levels at the end of the process. Previous work has demonstrated the importance of an oxidizing environment for proper disulfide bond formation, which may be particularly important for enzymes such as PDI that facilitate antibody disulfide bond formation (Hatahet and Ruddock, 2009). The above results clearly illustrate the importance of the redox environment in cell culture for improving both specific productivity and product quality. Although not shown in the current study, different components were individually screened for their effects on aggregates and productivity in
‘Medium 1’ and based on the results from the study, ‘Medium 2’ was subsequently designed. As the ‘redox modifying agents’ in ‘Medium 2’ contained more than one component, future work could involve studying the intracellular effects of individual components to ensure that observed results are not due to interactions between components. Taken together, the results shown in this article allow a better understanding of the intracellular response to process changes, and this understanding can help guide future process development for greater productivity and improved product quality.

Acknowledgements

The authors would like to acknowledge Mao-shih Liang for assistance with specific OUR calculations and Morenje Mlawa for assistance in sample purification. The authors also acknowledge Chris Sellick and Limin Qu in reviewing the manuscript and Madhukar Dasika in reviewing the amino acids analysis. Finally, the authors have no conflict of interest to declare.

References


Tables and Figure Legends

**Table 1:** Process Conditions and Outputs. The described processes were in duplicate 3L bioreactors.

**Figure 1:** Impact of Process Changes on Cell Culture Performance. The processes were run in duplicate 3L bioreactors and were inoculated at 1e6 cells/mL. Samples were taken on the indicated days to measure (A) viable cell density (VCD), (B) cell viability, (C) titer, (D) osmolality, (E) productivity, (F) cell culture redox potential, (G) lactate concentration, (H) ammonia concentration, and (I) total aggregate levels in cell culture supernatant. Data represents the mean ± s.d. from duplicate reactors.

**Figure 2:** HC and LC mRNA Expression. Heavy and Light chain mRNA expression were measured in cell pellets taken from each reactor on days 0 and 10. Data represents the mean ± s.d. from duplicate reactors.

**Figure 3:** Antibody Expression and Aggregate Analysis. (A) Cell pellets were collected on days 0 and 10 for intracellular western blots analysis of heavy and light chain expression under reducing conditions (top) and non-reducing conditions (bottom). (B) Cell culture supernatant samples were analyzed via western blots analysis for heavy and light chain expression under reducing conditions (top) and non-reducing conditions (bottom).

**Figure 4:** PDI Expression. (A) Cell pellets were collected on days 0, 6, 8, 10, 12, and 14. The pellets were lysed and the expression of PDI was determined via western blot analysis. (B) PDI expression was quantified relative to the expression of α-tubulin via densitometric analysis.
**Figure 5:** Amino Acid Consumption. The specific consumption rates of each amino acid were calculated during the mid-run phase of the cell culture processes (days 6-11). A downward arrow indicates that the amino acid was depleted at least once during the process. Data represents the mean ± s.d. from duplicate reactors.

**Figure 6:** Glucose, Lactate, and OUR. The specific (A) glucose and (B) lactate consumption rates were determined for three phases of the cell culture, growth (days 0-6), mid-run (days 6-11), and end of run (days 11-14). (C) The specific OUR was determined for each day of the cell culture process. Data represents the mean ± s.d. from duplicate reactors.

**Figure 7:** Oxidative Stress. (A) The relative intracellular concentration of H$_2$O$_2$ was measured via flow cytometry on days 0, 3, 5, 7, 10, 12, and 14. (B) Glutathione was extracted on days 3, 6, 10, and 14 from cell pellets. The ratio of reduced to total glutathione was analyzed using commercially available kits. (C) Relative level of total intracellular glutathione extracted from cell pellets on days 3, 6, 10, and 14. Data represents the mean ± s.d. from duplicate reactors.

**Figure 8:** Effect of Media on Aggregate Formation. The purified mAb was spiked in medium 1, medium 2, conditioned medium 1, and conditioned medium 2. The samples were incubated for 2 days at 36°C and aggregates were analyzed via SEC.
Table 1: Process Conditions and Outputs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Process A</th>
<th>Process B</th>
<th>Process C</th>
<th>Process D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>35.5</td>
<td>35.5</td>
<td>35.5</td>
<td>36.0</td>
</tr>
<tr>
<td>pH ± Deadband</td>
<td>6.80 ± 0.10</td>
<td>6.80 ± 0.10</td>
<td>6.80 ± 0.10</td>
<td>6.90 ± 0.10</td>
</tr>
<tr>
<td>Dissolved Oxygen (%)</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Agitation (RPM)</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Media</td>
<td>Medium 1</td>
<td>Medium 1</td>
<td>Medium 2</td>
<td>Medium 2</td>
</tr>
<tr>
<td>Glucose Strategy</td>
<td>Maintain glucose concentration &gt;1g/L for the duration of the process</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feed Type</td>
<td>Feed X</td>
<td>Feed Y</td>
<td>Feed Y</td>
<td>Feed Y</td>
</tr>
<tr>
<td>Feed Timing</td>
<td>Bolus feeds every other day starting on day 1</td>
<td>Bolus feeds every other day starting on day 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Titer (g/L)</td>
<td>4.3</td>
<td>9.0</td>
<td>7.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Specific Productivity (pg/cell-h)</td>
<td>1.1</td>
<td>2.1</td>
<td>2.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Peak VCD (1e6 cells/mL)</td>
<td>11.0</td>
<td>18.9</td>
<td>14.5</td>
<td>16.3</td>
</tr>
<tr>
<td>Aggregates (%)</td>
<td>21.8</td>
<td>21.2</td>
<td>18.0</td>
<td>16.9</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 4
This article is protected by copyright. All rights reserved
Figure 6
Figure 7
Figure 8