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Proteomic Analysis of Bioreactor Cultures of an Antibody Expressing CHO-GS Cell Line that Promotes High Productivity

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Abstract

Antibody manufacturing cell line development at Janssen Research & Development involves transfection of therapeutic antibody genes into a CHO-GS host cell line and isolating primary transfectomas that upon cloning yield high expressing cell lines secreting the desired antibody products. Subsequently, these cell lines are cultivated in stirred tank bioreactors for the large-scale generation of the products. In an attempt to optimize this process for high productivity, a two pronged approach was undertaken.

First, in a Design of Experiment study, a CHO-GS cell line expressing a therapeutic antibody was cultivated in 2 L DasGip fed-batch mini-bioreactors under a variety of culture conditions. In general, culture conditions that promoted robust growth and high viable cell density resulted in high productivity. Then, cell culture harvests and cell lysates from two 'high productivity' and two 'low productivity' bioreactors were subjected to proteomic analysis using the CHO genome database, on two independent days. The levels of each protein expressed in these two sets of bioreactors were then compared. A total of 180 proteins that were modulated two-fold or more were thus identified, only 12 of which were consistently modulated across multiple days in culture. The modulated proteins have biological process functions that are related to cytoskeleton rearrangement, protein synthesis, cell metabolism and cell growth. Provided that these observations are validated by Western blot, one or more of these proteins, whose expression correlated to productivity can potentially be utilized as targets for manipulating a superior transfection host cell line. At a minimum, the expression levels of these proteins can provide insight for further process optimization efforts.

Keywords: Antibody; Proteomic analysis; Production cell line; CHO-GS; DasGip mini-bioreactors

Abbreviations: Ab: Antibody; F8: Media Supplement; G8: F8 with Glucose; BRX: Media Supplement; Mach-1: Basal Media; D: Day; IVCC: Total Cell Count (Integrated Viable Cell Count); CL: Cell Lysate; SP: Supernatant; H-ch: Heavy Chain; L-ch: Light Chain; IT: Ion Trap

Introduction

Recombinant proteins including recombinant monoclonal antibodies (Abs) have been widely used for diagnostic and therapeutic purposes, and the demand for their supply is steadily increasing [1]. To meet this demand, a variety of strategies have been employed to develop efficient biopharmaceutical manufacturing processes. These strategies include: 1) maximizing the productivity of the manufacturing cell line by the judicious use of expression systems for optimal transcription and translation of the therapeutic protein [2]; 2) engineering the transfection host cell line for efficient post-translational modification and secretion [3]; and 3) improving the cell culture process including media optimization [4-8], developing advanced feeding strategies [9,10] which in turn increases the culture density. Additionally, robust and highly productive host cell lines can be deployed by increasing the efficiency of gene expression that regulates proliferation [11] survival and longevity [12-14]. Finally, methods that allow early prediction of unstable and stable cell lines by measuring intra-cellular Ab content for example, by flow cytometry [15,16] are highly desirable.

In our attempts to improve productivity of manufacturing cell lines, we had examined previously the titer and heavy chain (H-ch) and light chain (L-ch) mRNA copy numbers of a large number of mouse myeloma cells lines expressing a variety of antibody (Ab) products [17]. Results suggested that overall, there was a correlation of L-ch and especially H-ch mRNA levels to Ab productivity. However, this correlation was not very tight. A subset of cell lines that had increased Ab titers showed enhancement of H-ch and L-ch transcript levels, while another subset of cell lines showed minimal or no increase of these transcripts. Possible mechanisms by which the latter set of cell lines showed enhancement of Ab titers included: 1) increased stability of production cell lines [18-20], decreased apoptosis [21,22]; 2) increased secretion of Abs via chaperones [3] and 3) improved metabolic state of the cell line [23,24] that might lead to an increased proliferation of the cells and/or 4) prolongation of the G1 phase where protein synthesis is particularly more efficient [25].

The clonal variation in expression led us to hypothesize that there might be a correlation between the levels of one or more host cell proteins and Ab productivity. The presence of any particular protein thus identified could result from the Ab production by that cell line or be related to a pathway that can cause high Ab productivity. Alternatively, it is possible that the identified proteins are concurrently expressed with genes that lead to high productivity. Irrespective of whether it is the cause or the effect, a correlation between Ab

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productivity and the differential expression of one or more host cell proteins would suggest that, at a minimum, these proteins can be used as diagnostic markers for higher expressing cell lines. Additionally, in cases where the expression of one or more cellular proteins is the cause of higher Ab productivity, appropriate modulation of their levels could potentially lead cell lines with superior productivity. To determine if such a correlation exists, we cultured a cell line under a variety of media formulations. The productivity (or titer) obtained from all of these culture conditions were averaged and culture conditions that resulted in higher than average or lower than average productivity were identified. We then performed proteomic analysis of these cultures to identify proteins whose expression was correlated to culture conditions that promoted higher than average productivity as well as culture conditions that promoted lower than average productivity.

The development of shotgun proteomic technique provides a powerful tool to comprehensively determine the changes in protein expression levels in multiple cell lines or one cell line under multiple conditions. 'Global protein expression profiling using shotgun proteomics has been used in our laboratory to determine the protease profile of the CHO-GS host cell line [26] as well as gene expression profile of high producing cell lines [27]. Genome, transcriptome and proteome analysis of CHO cells have been reported multiple times [28-33]. Unfortunately, in a majority of these studies, the cells were cultured in shake-flasks prior to analysis. Since a shake-flask environment does not mimic a stirred tank fed-batch bioreactor that will be used for manufacturing the product, in the current study, we decided to cultivate a CHO-GS cell line expressing a therapeutic antibody in a set of eight fed-batch bioreactors. The composition of the basal medium and the feed was varied in each bioreactor. Cell growth, productivity and key metabolic indicators like the gradual accumulation of lactate and ammonia were monitored daily. Some of the bioreactor conditions led to higher productivity over other conditions. Proteomic analyses were performed on two duplicate sets of cultures on two different days. The level of each protein in cultures that promoted high productivity was compared to that in cultures that promoted low productivity.

Materials and Methods

Cell culture

The CHO host cell line, CHO-K1SV was obtained from Lonza Biologics (Slough, UK) under a commercial license agreement. This line was cultured in a proprietary chemically defined medium or CD-CHO medium (Invitrogen, Carlsbad, CA; cat # 10743011) supplemented with 6 mM glutamine in shake flasks and in 2 L DasGip mini-bioreactors (DasGip, an Eppendorf Company, Jülich, Germany). Shake-flask cultures were monitored daily and bioreactor cultures were monitored continuously, unless otherwise noted.

Development of production cell lines

An exponential culture of CHOK1SV cell line was electroporated with vectors (designed to express a therapeutic antibody) using an electroporator purchased from BTX Instruments, Holliston, MA. Manufacturer's recommended protocol was used. Two days post-transfection, cells were placed in GS selection media, composed of glutamine-free CD-CHO medium fortified with GS supplements (SAFC Biosciences, Lenexa, KS; Cat No. 58672) and 25-50 µM Methionine Sulphoximine, MSX, (Sigma; St. Louis, MO; Cat No. M3443). Individual parental clones (and subsequently, subclones) were isolated by the immunoprecipitation cloning method, and expanded in shake flasks in the same medium. Volumetric productivity of each culture was monitored daily by measuring Ab concentration in spent culture harvest, usually till D14. The selected cell line was expanded in 2 L DasGip mini-bioreactors.

Bioreactor cultures of the production cell line

A proprietary chemically defined basal medium (termed Mach-1), supplemented with growth enhancers (termed F8) served as the seeding media. The bioreactors were inoculated at 4×10^5 exponentially expanding cells/ml and from D3 to D12, were fed daily, a mixture of key amino acids and vitamins (termed BRX), at a rate of 4% of the bioreactor volume. Additionally, a formulation containing glucose and seven critical amino acids (termed G8) were fed daily, from D3 to D17, beginning with a rate of 0.07% and ending with a rate of 1% of the bioreactor volume. Temperature was maintained at 36.5°C, pH was maintained at 6.9 by using 2 M sodium carbonate, agitation was set at 120 rpm, pCO2 was maintained at 6% and pO2 was set at 40%. Cultures were monitored daily by withdrawing samples for the following measurements:

- Total and viable cell count was measured by Cedex (Innovatis, GmbH). Integrated viable cell count [IVCC] on any given day was calculated thus: IVCC [dn]=[VCD [dn-1]+VCD [dn]]/2+VCD [dn-1]/cell-day where, VCD is viable cell density and dn is day of measurement
- Overall volumetric productivity of a culture, also referred to as the titer is dependent on the IVCC of the culture and the specific productivity (Qp) of the cell line.
- Antibody concentration (i.e., titer) in culture fluid was measured by nephelometry (Protein Array, Beckman Instruments, Fullerton, CA).
- Metabolites (specifically, glucose, NH4, lactate, glutamine, glutamate, osmolarity) in the culture medium or spent culture fluid were measured by Bioprofile Analyzer (Nova Biomedical, Waltham, MA) and pH and pO2 were measured by Blood Gas Analyzer (Bayer, Montvale, NJ). CO2 was monitored by IR sensor.

Preparation of spent media and cell lysates

At designated days, aliquots of the selected bioreactor cultures were harvested for proteomic analysis. Spent media (SP) were clarified by centrifugation at 900rpm for 5 min. The cell pellets (comprising of ~2×10^8 viable cells) were used for the cell lysates (CL). The cells were washed twice with equal volume of PBS to remove residual medium and other minor contaminants. Two milliliters of RIPA buffer (Sigma-Aldrich; cat # R0278) containing cocktails of protease inhibitors (G-BioSciences, St. Louis, MO; cat # 786-108) and phosphatase inhibitors (Sigma; cat # P8340) were added to each cell pellet and mixed gently. The mixture was incubated on ice for 10 min, at which time, the cells were lysed and the organelles were completely solubilized by the detergents present in the RIPA buffer, namely, sodium deoxy-cholate and NP40. The CL samples were clarified by centrifugation at 8000g for 10 min at 4°C. Both the SP and CL samples were stored at -80 °C till further use.

SDS-PAGE purification of host-cell proteins

The host cell proteins present in the SP samples as well as the CL samples had the therapeutic protein as a major contaminant. Therefore, these samples were partially purified by SDS-PAGE. Fourteen microliter of SP samples (up to 50 µg of protein) or 5 µl of

CL samples was mixed with 5 µL 1M DTT and 6 µL NuPAGE LDS sample buffer (4X) (Invitrogen, cat # B0007) in a final volume of 25 µL. The mixture was incubated at 90°C for 10 min, and then the entire content was loaded onto one lane of a 10-well Novex 4-12% Bis-Tris SDS-PAGE gel (Invitrogen; cat # NP0301BOX). The gel was subjected to electrophoresis using MES-SDS running buffer (Invitrogen; Cat # NP002). The gel was then stained with Coomassie blue. The host cell proteins in each lane were excised as five gel sections and each gel section transferred to fresh tubes, carefully avoiding the H-ch and the L-ch peptides of the therapeutic Ab that the cell line is expressing. This procedure removed a substantial portion but not all of the H-ch and L-ch contaminant.

In gel trypsin digestion of proteins

The proteins in each gel section described above underwent in-gel trypsin digestion. Each gel section was minced (approximately 0.5 mm^2) and transferred to a microcentrifuge tube and subjected to 2 to 3 cycles of gel dehydration with acetonitrile and rehydration with ammonium bicarbonate buffer (0.1 M, pH 8.0) in order to remove the Coomassie stain. The destained gel slices were then reduced by the addition of 250 µL of 10 mM DTT in 0.1 M NH_4HCO_3 and incubated for 30 min at 56°C. The samples (gel slices) were subsequently alkylated at room temperature and in the dark for 60 min with 250 µL of 55 mM Iodoacetic acid (Sigma, cat # PROTRA) in 0.1M NH_4HCO_3. After removal of the liquid, 250µL of trypsin digestion solution (containing 10 ng/µL trypsin (Sigma, cat # T4434) in 50 mM NH_4HCO_3, pH 8.0) was added to the gel slices, and the samples were then incubated for 30-35 min at 37°C. The incubated solution was removed and saved. Sufficient quantity (50-100 µl) of 50 mM NH_4HCO_3 was added to cover the gel pieces and then incubated overnight at 37°C. The two supernatants were combined. The gel pieces were further extracted with 5% formic acid (100 µL) at 37°C for 5 min, and an equal amount of acetonitrile was subsequently added, and the sample was shaken for 15 min at room temperature. The formic acid and acetonitrile solution, containing tryptic peptides, were combined with the previous supernatants and concentrated. The volume was then adjusted such that the peptide concentration was 0.7 µg/µl and aliquot of 5 µL of the samples was used for subsequent LC-MS analysis.

LC-MS analysis

LC-MS experiments were performed on an online nano-LC instrument coupled with LTQ-FT (Thermo Fisher Scientific, San Jose, CA). LTQ-FT combines a linear ion trap and Fourier transform ion cyclotron resonance (FTICR) mass analyzers. The LC system was composed of an Ultimate 3000 nano-LC pump (Dionex, Moutain View, CA) and a self-packed capillary C18 column (Magic C18, 200Å pore size and 5 µm particle size, 75 µm i.d×10 cm) (Michrom Bioresources, Auburn, CA). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The sample loading was 5 µL (0.7 µg/µl). The analytical separation was carried out by a linear gradient: starting from 2% B to 40% B in 40 min, increased to 60% B in 10 min, and then to 80% B in 5 min. The column flow rate was maintained at 200 nL/min. The instrument was operated in the data-dependent mode: the first survey MS (scan 1) from m/z 400 to 2000 followed by three consecutive ion activation steps: CID-MS2 (scan 2), CID MS2 (scan 3), and CID-MS3 (scan 4). The survey full-scan MS spectra with two microscans (m/z 400-2000) were acquired in the Fourier transform ion cyclotron resonance cell with mass resolution of 100,000 at m/z 400 (after accumulation to a target value of 2×10^6 ions in the linear ion trap), followed by sequential LTQ-MS^2 scans. Dynamic exclusion was utilized with exclusive duration of 30 sec and no repeat counts. The total cycle time (1 FTICR survey scan with two micro scans plus eight sequential linear ion trap MS2 scans) was 2.7 sec.

Database search

The proteins were identified by Thermo Scientific Proteome Discoverer 1.3. The Sequest search algorithm was used to search MS/MS data against a publicly available Chinese hamster ovary (CHO) database. The database was downloaded from http://www.chogenome.org in December 2012 [34-36]. The five MS raw data generated from the five digestion sections of each sample were uploaded together and the search results were combined for each sample. The search parameters were 50ppm processor ion tolerance, 1.5 Da fragment mass tolerance and Cys carbamidomethlyation static modification. The filter and protein grouping settings were high peptide confidence-value and 1 maximum peptide rank. The absolute cross correlation score (XCorr) threshold of peptide scoring was 0.4. The fragment ion cutoff percentage was 0.1. The XCorr cutoffs were 1.2, 1.9, 2.3, and 2.6 for peptides with charge states of +1, +2, +3, and ≥ +4 respectively. All proteins were mapped to their genes using NCBI Entrez database [http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi] for information regarding annotating the identified proteins with respect to gene ID, symbol and name.

Data manipulations

In order to determine correlation of one or more CHO cell proteins to volumetric productivity, two high productivity bioreactors [R10H021 and R10H022] and two low productivity bioreactors [R10H020 and R10H023] were selected. Each set of bioreactors were considered as biological replicates (with respect to productivity) even though each bioreactor had a unique set of culture conditions.

Normalization: Because of the potential opportunity to introduce errors in sample preparation and spectrometry assays, the peptide number (obtained from the spectral count) of each protein in each dataset was normalized. A set of house-keeping proteins whose expression level was high and known not to be affected by varying bioreactor culture conditions, were selected for this purpose ([37], Table 2).

Statistical analyses: Following normalization, the data was subjected to imputation and transformation for statistical analysis. For this purpose, +1 was added to each of the peptide number in each protein in each sample (data imputation). The minimum value thus became 1 instead of zero. Then Log2 transformation was applied followed by another round of imputation. Next, the ratio of each protein was calculated from its normalized peptide count in high productivity bioreactors as compared to that in low productivity bioreactors. For each ratio, p-values were calculated using the general linear model (GLM) procedure of ArrayStudio 6.1 (http://www.omicsoft.com/arraystudio.php). Data were fitted into a linear model with productivity, time and the interaction between the two. Proteins which were up-regulated 2-fold or more or down-regulated 2-fold or less and had a p-value of less than 0.125 were considered further. This p-value and fold-cutoff were selected to generate a working list of proteins for each set of bioreactors.

Results

In our aim to improve productivity (specific/volumetric) of therapeutic products manufactured in mammalian expression systems, we undertook a two-pronged approach. First, we systematically analyzed the cell culture media components that impact the

metabolic state of the cell line. An optimized combination of these components is expected to lead to an increased proliferation of the cells, which in turn will lead to higher volumetric productivity, even if the specific productivity of the cell line remains unaltered. Similar studies performed in other laboratories have relied on terminal batch cultures in shake flasks [28,32,33], but since cell culture conditions in shake flasks do not necessarily mimic those in bioreactors, and since the manufacturing step is generally carried out in bioreactors either in fed-batch or in perfusion mode, we have performed all our studies in 2L mini-bioreactors in the fed-batch mode. A CHO-GS cell line expressing a therapeutic Ab was cultured in eight independently controlled DasGip CellFerm Pro mini-bioreactors using an 18-day fed batch process. The effect of various media components on growth and volumetric productivity was investigated by a DOE study using 2^{5-2} resolution, three-quarter-factorial "screening" design. With this design, the impact of increasing or decreasing the concentration of each of the four media components (labeled Mach-1, F8, BRX and G8, Table 1A) are investigated and each effect is aliased with a 2-way interaction in a total of eight bioreactors.

Impact of the basal and feed media on viability and titer

Overall productivity of a culture (i.e., its titer) depends on IVCC, (which in turn depends on viable cell count) and the specific productivity (Qp) of the cell line. Figure 1 shows the viable cell count and the total cell count of the eight bioreactor cultures across all 18 days. As seen in this figure, all eight bioreactors trended similarly up to D9. From D11 onwards, the viability of the cultures began to diverge from each other, being dependent, in part, on BRX concentration; lower amounts of BRX resulted in comparatively lower viability while higher amounts of BRX resulted in higher viability. For example, R10H017 which was fed 0.85X (or 85% of the standard amount) of BRX had 20% viability

Reactor Number (ID)	Mach-1	F8 (g/L)	BRX	G8
1 [R10H016]	0.9X	8	0.85X	1.15X
2 [R10H017]	1.1X	8	0.85X	0.85X
3 [R10H018]	0.9X	8	1.15X	0.85X
4 [R10H019]	0.9X	6	0.85X	0.85X
5 [R10H020]	1.1X	6	0.85X	1.15X
6 [R10H021]	0.9X	6	1.15X	1.15X
7 [R10H022]	1.1X	6	1.15X	0.85X
8 [R10H023]	1.1X	8	1.15X	1.15X

Table 1A: A CHO-GS production cell line was cultured in eight independently controlled two liter mini-bioreactors (DasGip, CellFerm-pro). These bioreactors, labeled R10H016 to R10H023 were set up according to Table 1, 1X being the standard condition described below and in Materials and Methods. To determine the impact of media components on productivity, a DOE study (three-quarter-factorial "screening" design) using 2^{5-2} resolution was implemented. With this design, the effect of four media components, labeled Mach-1, F8, BRX and G8 was investigated. Each bioreactor received the indicated fraction of a standard bioreactor condition.

Standard bioreactor condition, defined as 1X for this study, is as follows: 806ml of growth media, which is a mixture of a proprietary chemically defined basal media termed Mach-1 supplemented with 8 g/L of a proprietary formulation of growth enhancers, termed F8, was seeded with 94 ml of inoculums (0.4×10^6 cells/ml), for a total volume of 0.9L. From D3 through D12, a proprietary formulation of vitamins and amino acids, termed BRX, was fed at a daily rate of 4% of the bioreactor volume. Additionally, from D3 through D17, a proprietary formulation of glucose and a select set of rapidly utilized amino acids, termed G8, were fed starting with 0.07% on D3 and ending with 1% on D17.

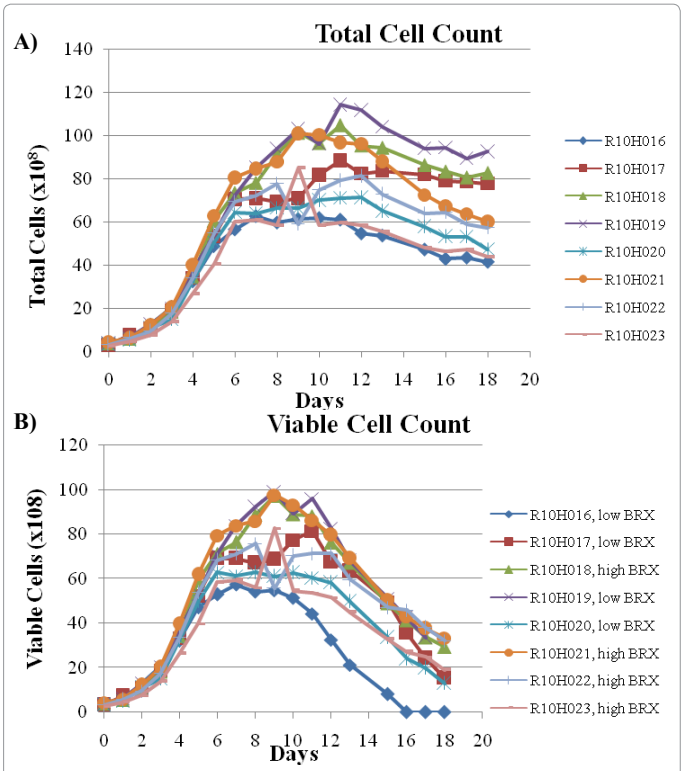

Figure 1: The effect of media component, BRX, on the viability and viable cell count (VCD) of a CHO-GS cell culture. Each bioreactor was run with two different concentrations, either 'low', 'standard' or 'high' of basal media (termed Mach-1 and F8) and two different concentrations of feed medium (termed G8 and BRX), Standard condition for F8 is 8 g/L whereas 'low F8'concentration is 6 g/L (Table 1). A) Viable cell count and B) Total cell count.

on D18, whereas the viability of R10H023, which was fed 1.15X BRX was about 60% at about the same time. This relationship between BRX and viability can be explained in part by the fact that when the cells were fed the lower concentration of BRX, higher concentrations of accumulated lactate was observed between D11 to D18 (Figure 2), a cell culture waste that is cytotoxic to most mammalian cells. For example, bioreactor R10H016, which was fed 0.85X BRX, the concentration of lactate reached about 7 g/L, whereas the bioreactor R10H021 which was fed 1.15X BRX, had accumulated only 1.8 g/L lactate during equivalent period of time. Not surprisingly, there was a sharp decline in viability in R10H016 beginning as early as D9 (and reaching 40% viability on D12) whereas, R10H021 had the highest viability (retaining 58% viability on D18). Consequently, as shown in figure 3, R10H016 had the lowest product titer (747 mg/L) whereas R10H021 had one of the highest product titers (2258 mg/L).

In order to determine the relationship between the various media components and the various cell culture parameters, the D9 data was graphed (Figure 4). The four columns show the data generated from bioreactors that had Mach-1, F8, BRX and G8 respectively. The five rows show the data for the five cell culture parameters, which included accumulated lactate, accumulated NH4+, glucose consumption, percent viability and volumetric productivity. Results suggest that 1) Mach-1 had a small effect on glucose consumption level; 2) F8 had a significant effect on the lactate levels; 3) BRX had a significant effect on lactate levels and viability; 4) G8 had a significant effect on the amount of glucose consumption level as well as viability; 5) the accumulation

Bioreactor	R10H016	R10H017	R10H018	R10H019	R10H020	R10H021	R10H022	R10H023
IVCC (×10^9 cell-day)	41.6	78	82.8	92.9	47.5	60	57.5	44
Titer (mg/L)	747	1816	2061	1996	1448	2330	2258	1702
Qp (pg/cell/day):	15.3	23.3	24.9	21.5	30.5	38.6	39	38.7

Table 1B: Specific productivities of a CHO-GS production cell line cultured under various culture conditions.

of ammonia was not impacted by any of these components and 6) importantly, productivity was indirectly impacted when viability was impacted. Table 2B shows the effect of the eight bioreactor conditions on the IVCC, titer and the specific productivity of the CHO-GS cell line. It varied from 15.3 pg/c/day for R10H016 to 39 pg/c/day in R10H022.

Proteomic analysis of the bioreactor samples

In the second part of our study, in order to determine if there is a correlation between overall productivity of a culture and the levels of intra-cellular and/or secreted proteins, CL and SP samples from two high productivity/high viability bioreactors [R10H021 and R10H022] and two low productivity/low viability bioreactors [R10H020 and R10H023] were selected for proteomic analyses. (Even though R10H016 had the lowest titer, we disregarded this bioreactor due to the precipitous loss of viability for possible unknown causes). Cell cultures from the above four bioreactors were harvested on D7, D11 and D15 and 24 samples comprising of 12 CL samples and 12 SP samples were prepared for analysis.

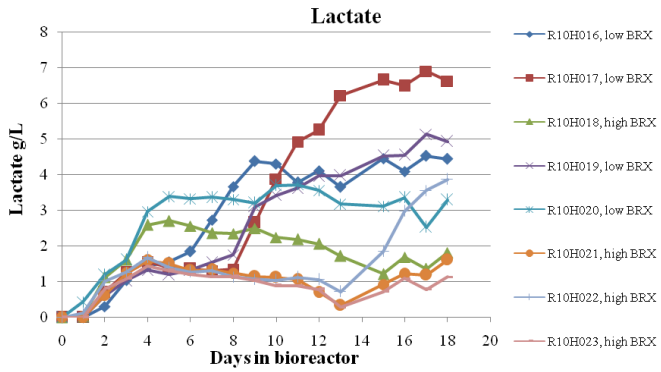

Figure 2: The effect of media component, BRX, on the accumulation of lactate byproduct in culture harvest.

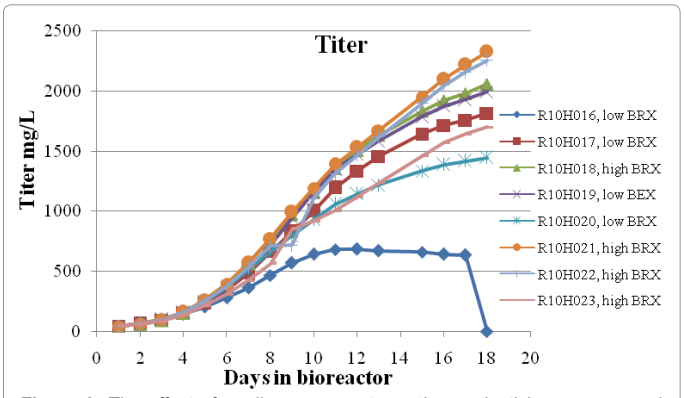

Figure 3: The effect of media components on the productivity as measured by titer.

Analyses of the LC-MS data

The above 24 bioreactor samples, following purification and tryptic digestion, were subjected to LC-MS analysis, which allowed the identification of a majority of the proteins present in each sample, is shown in 24 tables in table S.I, datasheet A, in the supplemental data section. Each table lists all the proteins identified in each of the 24 bioreactor samples. Each protein is identified by its unique accession number (GI; column 1) followed by the name of the protein (column 2). The abundance of each protein, which has been shown to be correlated to the number of peptides generated for that protein [38,39], is shown in column 3. From this data, we concluded that a reduced number of proteins were being detected in D7 samples, especially those that are being secreted in the media. Hence data generated from D7 samples were not considered in subsequent studies, thus reducing the number of samples to eight CL and eight SP samples.

The accessions numbers (GIs) of all the proteins that have at least one peptide in any of the 16 samples were collected into a single data matrix (compiled in Table S.II, datasheet A). The resulting 2890 unique GIs were mapped to Entrez gene databank for information on annotation with respect to gene ID, gene symbol, protein name and its function. In this table, each protein is identified by its unique accession number (column A), and Entrez ID of the gene coding the protein (column B), followed by the symbol and name of the gene (column C and D). Table S.II, datasheet B lists the original peptide count (obtained from the spectral count) of each protein in each sample. Note that there were many potentially important proteins that were detected only in some but not all bioreactor conditions. For example, protein GI#354493414 was detected in a high productivity bioreactor on D15, but not on D11 (Table S.II, datasheet B). In these cases, data imputation, i.e., addition of +1 to the peptide count of each protein raised the minimum value of all peptide counts from zero to one, thus enabling calculation of ratios for these proteins as well. Table S.II, datasheet C shows the peptide count of each protein in each sample after normalization as described in Materials and Methods.

Under the assumption that the samples from the set of two high productivity bioreactors are biological replicates, the normalized peptide count of each protein derived from R10H020 and R10H023 were averaged, as also that derived from the set of two low productivity bioreactors, namely, R10H021 and R10H022. The average peptide count of each protein detected in the high productivity bioreactors [R10H021 and R10H022] was compared to that in low productivity bioreactors [R10H020 and R10H023] resulting in the generation of a ratio for each protein on D11 and on D15, in CL and SP samples (Table S.II, datasheet D). As expected, the data identified several proteins that were highly up-regulated or highly down-regulated, with the levels of a vast majority of the remaining proteins either unchanged or modulated to a smaller extent. Proteins that were up-regulated 2-fold or higher (ratio ≥ 2.0) or were down-regulated 2-fold or lower (ratio ≤ 0.5) were selected for further study. The selected proteins could be grouped into four categories: 1) intracellular and up-regulated, 2) intra-cellular and down-regulated, 3) secreted and up-regulated

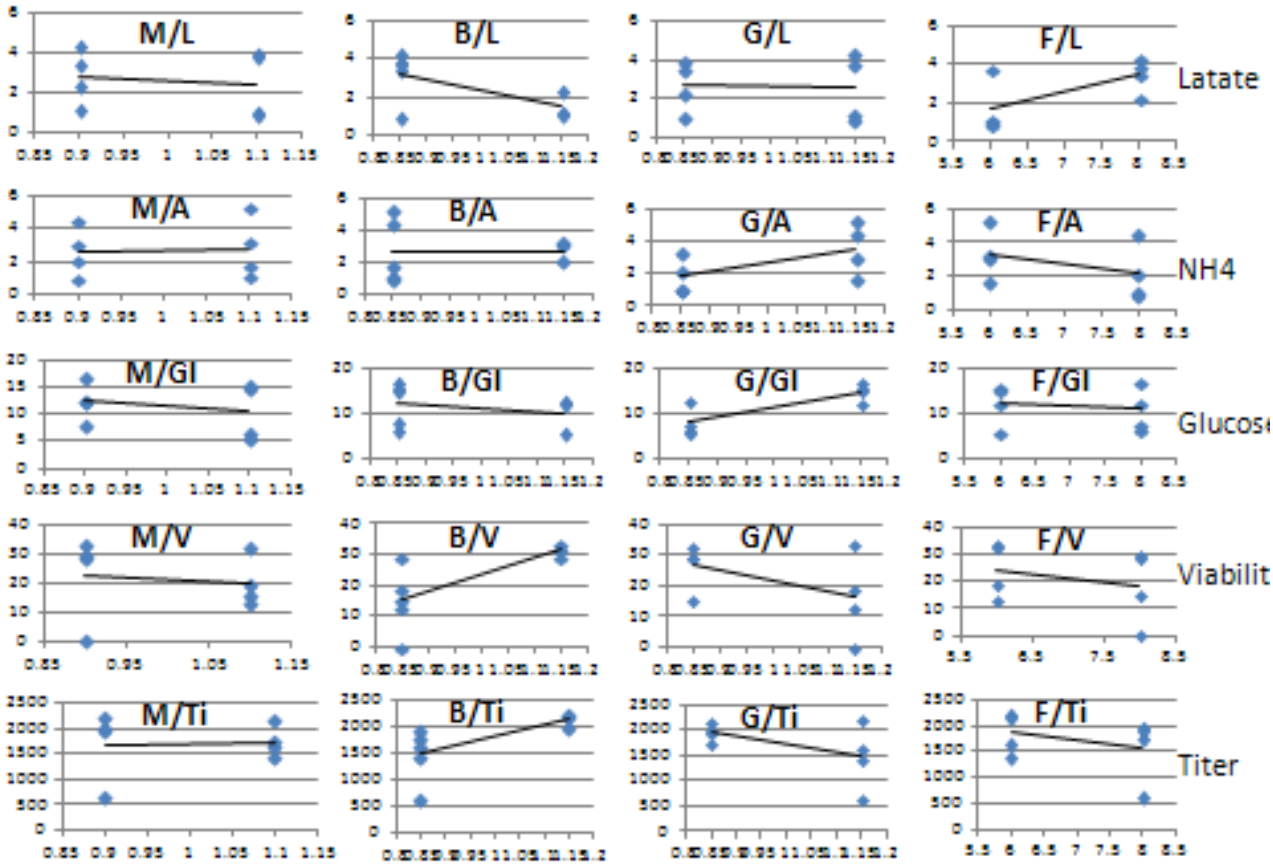

Figure 4: The effect of four different media components on multiple cell culture parameters. Eight mini-bioreactors were set up using the media formulations outlined in Table 1 and in Materials and Methods. A number of cell culture parameters were monitored daily, including glucose consumption, lactate and ammonia accumulation, viability and titer. The data generated on D9 is graphed. The four columns show the 4 different media components, namely, Mach-1 (M), column 1 from left, BRX (B), column 2, G8 (G), column 3 and F8 (F), column 4. The 5 rows refer to the cell culture parameters, namely, Lactate (L), Ammonia (A), Glucose (Gl), Viability (V) and Titer (Ti).

and 4) secreted and down-regulated. The lists of proteins that fell in these four categories are shown in Table S.II.1 through S.II.4 (Table S.II, Datasheet E). Data generated from D11 and D15 samples in each of these four categories are shown as parts A and B, for each of the above four tables, respectively. Each protein in each table has a unique accession number, followed by the gene symbol, the description of the protein and the ratio of its expression level as detected in high' vs. 'low' productivity bioreactors. Thus, a total of 180 proteins were identified to be up-regulated two-fold or more and 97 proteins were down-regulated two-fold or more. Of the 180 proteins that were up-regulated on D11 and/or on D15, 47 proteins were detected in the SP samples and 133 proteins were detected in the CL samples. Of the 97 proteins that were down-regulated, either on D11 and/or on D15, 37 proteins was detected in the SP samples and 60 proteins were detected in the CL samples (Table 3A). Within each category, we next determined which of the proteins were present in both D11 and D15 by merging the D11 and D15 data of each category (see Table S.II, datasheet E). Proteins that were similarly modulated on both on D11 and D15 were bolded and highlighted in yellow. This procedure was repeated for the remaining three categories and a list of all the proteins that were detected in both D11 as well as D15 was generated. As can be seen from table 3B, only twelve proteins are similarly modulated both on D11 and on D15. Additionally, 18 other proteins were identified where the members of the same family of proteins are similarly modulated on D11 and D15 (bolded). These proteins are listed in table 3C. With a few exceptions, the selected proteins and protein families can be grouped into four distinct functional groups, as determined using Gene Ontology database (www.geneontology.org), namely, 1) control of proliferation; 2) protein synthesis; 3) cytoskeleton rearrangement; and 4) maintenance of the metabolic state of the cell.

Discussion

Manufacturing cell line development at Janssen Research & Development involves transfection of therapeutic antibody genes into CHO-GS host cell lines and isolating primary transfectomas that upon cloning yield high expressing cell lines secreting the desired product. In an attempt to increase overall productivity of these cell lines, a number of investigations have been undertaken in the past several years. These investigations, which follow closely those published in the literature, include 1) determining the optimal copy numbers of H-ch and L-ch of the therapeutic Ab in the transfectomas [17,40]; 2) utility of apoptotic resistant host cell lines [13,23,24]; 3) increased secretion of Abs via over-expression of chaperones [3] (unpublished observation); 4) prolongation of the G1 phase where protein synthesis is particularly more efficient [25] and 5) judicious use of expression systems and cell line selection strategies [2,41,42]. Recently, the "omics" approach for

improving productivity has been embraced by a number of researchers [27,29,33]. Indeed, we have performed microarray analysis of selected myeloma production cell lines in order to determine if there is a correlation between expressions of one or more myeloma cellular genes to productivity [27].

In the last step of the cell line selection process, several candidate production cell lines are tested, ideally, in bioreactors, which is a critical but a very labor-intensive and a time-consuming process. In order to reduce cost and time in biopharmaceutical development, emphasis is on scale-down models and predictive technologies. However, the shake-flask culture, which is the widely used scale-down model of fed-batch bioreactor, does not mimic the environmental conditions of a stirred-tank, fed-batch bioreactor well [16,39]. Consequently, in our laboratory, the final step in the cell line screening process is routinely carried out in bioreactors. With that in mind, in the current study, we have used a two-step approach to improve productivity in a CHO-GS production cell line. First a DOE study to optimize the basal growth media components and feed composition of the production cell line was carried out. This was followed by a comparative proteomic analysis of the high vs low productivity cell lines. In the DOE study, the composition of the basal and the feed medium had a dramatic effect on the growth profile of the cell line as well as accumulation of common metabolic waste products like lactate and ammonia. The relationship between the components of the basal and that of the feed medium were complex. Cultures that had less than the standard amounts of BRX but increased amounts of G8 (proprietary formulation of glucose, vitamins and amino acids] resulted in an increase amount of lactate and NH4+ accumulation in the bioreactor. The two bioreactors that had the highest viability [R10H021 and R10H022] were initiated with lower amounts of the growth enhancing supplement F8 to slow down the initial rate of metabolism. This strategy of reduced feeding has been successfully tested previously [9,10]. In the current study, the reduced feeding is subsequently augmented by an increase in BRX feeding, such that high cell density is achieved. Consequently, the average IVCC of R10H021 and R10H022 were 150-157% higher than that of R10H020 (Figure 1 and Table 1B). Not surprisingly, improved growth profile and higher IVCC resulted in higher productivity as determined by measuring the titer of the cell culture harvest (Figures 1 and 3). Note that the specific productivity of the selected cell lines were not significantly different from each other, ranging from 26.6 pg/c-day to 36.4 pg/c-day (Table 1B).

In order to elucidate the mechanism of this increase in productivity, a proteomics approach to identify proteins whose modulation is correlated to high productivity was then undertaken. Proteomics approaches for improving productivity in CHO cells have been used previously [28-33]; specifically, a proteomics approach was used by Yee et al. [28] to study the effect of sodium butyrate on CHO cells, whereas Kaufmann et al studied the effect of lowering culture temperature on productivity [43]. While similar to these studies, there are several important features that distinguish the current study from the above published studies. Firstly, even though many manufacturing cell lines utilize the CHO-GS system, it has not been possible to study the CHO genome and proteome as the genome database was not publicly available until now (www.chogenome.org). Secondly, in the absence of a publicly available CHO genome database, specific CHO proteins were identified by 2D gel electrophoresis, MALDI-TOF analysis, N-terminal sequencing and cross-species database matching. Thirdly, with a few exceptions, these studies have been performed with cells cultured in shake flasks. While a great deal of knowledge has been gained from those studies, variability in dissolved oxygen concentration and consumption rate, metabolite profiles, and proteome was greater in shake flask than controlled batch or chemostat cultures. Proteins indicative of suboxic and anaerobic growth were more abundant in cells from shake flasks compared to bioreactor cultures, a finding consistent with data demonstrating that "aerobic" flask cultures were O_2 deficient due to poor mass transfer kinetics [44].

In the proteomic analysis, the abundance of each CHO protein derived from cells that were generated in bioreactors that had the highest viability and productivity [R10H021 & R10H022] was compared to that derived from cultures that had some of the lowest viability and productivity [R10H020 & R10H023]. Of the 2890 proteins detected in one or more of the varying bioreactor culture conditions, 277 proteins were observed to be modulated (two-fold or more), between high vs low productivity conditions and only 12 proteins showed consistent and reproducible modulation of expression on both D11 and D15.

There are several possible reasons why so few proteins were captured in our assay. 1) the bioreactors we have considered as biological replicates with respect to productivity have very different culture conditions, which might reflect in the overall pattern of gene expression; 2) due to the redundant nature of many of the biochemical pathways, there were many instances where similar (but not identical) proteins were modulated under the two different culture conditions; For example, Histone 3.2 was down-regulated in R10H020 and Histone 3.3 was down-regulated in R10H023; 3) A majority of the proteins that showed a significant modulation between different culture conditions were of the low abundance type, peptide count not exceeding 10 as compared to a peptide count of 90 for a protein considered of average abundance (Table S.II, datasheet B). A small change in the levels of the low abundance proteins result in a large impact on the fold-change calculations. High abundance proteins have to significantly alter their levels in order to achieve the same fold-change effect. Given the likelihood of selecting false positives is higher among low abundance proteins, we have instituted a number of checkpoints to avoid this problem. These checkpoints include 1) considering two bioreactors with similar productivities but with different culture conditions as biological replicates; 2) only using statistically significant data; 3) a minimum fold change of no less than 2-fold; and 4) selecting proteins that are expressed at both D11 and D15 . We speculate that since product secretion peaks after the cessation of exponential growth (Figures 1 and 3), proteins that enhance productivity should be consistently expressed both at D11 and D15. Also, the likelihood of selecting false positives would be lower. Western blot and RT-PCR experiments are essential to confirm the expression of the proteins that have been identified from this study.

The correlation between expression of selected proteins and productivity raises the intriguing possibility that these and other cellular proteins can possibly be utilized for eliminating the bioreactor screening step. Proteomic analysis data does not reveal whether the observed correlation is the cause or effect of high productivity. Further work needs to be done to determine the exact roles of these proteins in various cellular processes that contribute to the overall productivity of the cell lines. Irrespective of the molecular mechanism of the observed correlation, at a minimum, expression levels of these proteins can be utilized as surrogate markers for identification and development of cell lines with superior productivity. Moreover, these markers can be used for media and bioreactor process optimization steps.

The current study has utilized only one CHO-GS production cell

Lysates (CL)	R10H020			R10H021			R10H022			R10H023		
	D7	D11	D15	D7	D11	D15	D7	D11	D15	D7	D11	D15
Tubulin α-1B	18	17	18	20	19	16	16	20	16	21	19	16
Tubulin-β	20	23	20	23	21	21	19	23	20	24	23	18
Tubulin β-2C	18	21	16	22	21	19	18	22	18	23	22	16
actin-β	20	21	21	24	22	23	22	19	16	21	22	13
Gapdh	17	20	19	20	22	20	16	19	17	20	19	14
H-ch	19	21	23	20	21	21	20	20	20	22	20	20
L-ch	9	11	12	11	12	12	12	11	11	12	12	10
Sum	121	134	129	140	138	132	123	134	118	143	137	107
Norm. Factor	1	0.903	0.938	0.8643	0.8768	0.9167	0.9837	0.903	1.0254	0.8462	0.8832	1.1308
Supernatants (SP)	R10H020			R10H021			R10H022			R10H023		
	D7	D11	D15	D7	D11	D15	D7	D11	D15	D7	D11	D15
H-ch	36	40	41	36	37	42	38	40	41	40	38	42
L-ch	23	20	22	16	20	21	16	21	21	21	19	21
Sum	59	60	63	52	57	63	54	61	62	61	57	63
Norm. Factor	1	0.9833	0.9365	1.1346	1.0351	0.9365	1.0926	0.9672	0.9516	0.9672	1.035	0.937

Table 2: Normalization of the data generated from D11 and D15 bioreactor samples.

Top panel: Intra-cellular proteins; Bottom panel: Secreted proteins.
The peptide count of these selected proteins in each sample was summed up, and the sum obtained from H20D7_CL and H20D7_SP samples (121 and 59, respectively), were arbitrarily set as normalization standard, i.e., possessing a normalization factor (NF) of 1. Normalization of the proteins in other samples was accomplished by dividing the total peptide counts of these house-keeping proteins by that of H20D7_CL or H20D7_SP, for samples derived from cell lysates or culture supernatants, respectively. House-keeping proteins used for CL samples included glyceraldehyde-3-phosphate dehydrogenase, actin β, tubulin β, tubulin α-1β and tubulin β-2C. Additionally, the H-ch and the L-ch of the therapeutic protein being expressed by the cell were included in this normalization process. For the data generated from SP samples, the normalization process used only the H-ch and L-ch of the therapeutic ab being expressed by the cell line. This was necessary as the above house-keeping genes were either undetectable or present in very low levels in the SP samples. Even though the H-ch and L-ch protein bands were largely avoided during the sample preparation step (described above in the sample preparation section), sufficient residual quantities of both of these proteins were still retained in the sample to justify using them as housekeeping genes. Moreover, normalization with other structural proteins, e.g., Decorin, gave comparable results.

Table 3A	Number of proteins with 2-fold or more change			
	CL, Up-reg	CL, Down-reg	SP, Up-reg	SP, Down-reg
D11	65	38	40	19
D15	68	22	7	18
Total	133	60	47	37
Grand Total				277

Table 3A: A: Number of proteins being modulated (two-fold or higher) in one or both sets of bioreactors on D11 and/or D15. Green: Up-regulated and Red: Down-regulated. Both, intracellular (CL) and secreted (SP) were monitored.
B: List of intra-cellular and secreted proteins that were consistently and reproducibly up-regulated or down-regulated (two-fold or higher) in both D11 and D15 samples.
C: List of protein families that were consistently and reproducibly modulated in D11 and D15 samples. Different members of these protein families were up-regulated or down-regulated in D11 and D15 samples.

GENEID	Symbol	Description	D11	D15
D11 and D15, CL, Up-regulated			Fold change	Fold Change
100765619	LOC100765619	ADP-ribosylation factor protein 8B	2.58	2.79
100774665	LOC100774665	V-type proton ATPase subunit B, brain	2.56	2.04
100769573	LOC100769573	Uncharacterized protein KIAA0564	4.28	5.32
100768654	LOC100768654	retinal dehydrogenase 1	3.67	3.0
100767097	LOC100767097	cAMP-dependent protein kinase catalytic	4.47	3.0
D11 and D15, CL, Down-regulated				
100757036	LOC100757036	60S ribosomal protein L5	2.3	6.7
100767908	Me1	malic enzyme 1, NADP(+)-dependent, cy	2.9	2.5
100763574	Scfd1	Sec1 family domain containing 1	2.5	2.5
D11 and D15, SP, Up-regulated				
100767142	Csf1	colony stimulating factor 1 (macrophage)	2.56	6.17
100754832	Angptl4	angiopoietin 4	4	3
D11 and D15, SP, Down-regulated				
100767831	LOC100767831	inositol-3-phosphate synthase 1	4	3.6
100758958	LOC100758958	isocitrate dehydrogenase (NADP) cytoplas	2.3	2

Table 3B: Proteins that are modulated both on D11 and D15.

D11 and D15, CL, Up-regulated
ADP-ribosylation factors
Annexins
60S ribosomal proteins
Actin related proteins
Cyclin dependent kinases
Eukaryotic translational initiation factors
Flotillins
Glutathione S-transferases
Serine/arginine-rich splicing factors
Keratin type II
NADH dehydrogenases
proteasome subunits
D11 and D15, CL, Down-regulated
Heat shock proteins
Heterogenous nuclear ribonuclear proteins
Keratins
60S ribosomal proteins
D11 and D15, SP, Up-regulated
Collagen α-(IV)
D11 and D15, SP, Down-regulated
Serpins

Table 3C: Protein families that are modulated on D11 and D15.

line. It would be interesting to determine if one or more of the above 12 selected proteins are modulated in other CHO cell lines, or other CHO-GS production cell lines, making our findings more broadly applicable to the biologic pharmaceutical industry. It is possible that the observed modulation of the selected proteins in response to differential feeding is cell line specific and therefore will not serve as markers of productivity for other cell lines. However, the CHO-GS system, (licensed from Lonza) is highly popular and widely used by many biotechnology and pharmaceutical companies interested in the development of highly productive CHO-GS cell lines. Therefore, cell line specific findings, if any, will be useful for further work in this area. Moreover, the proteins identified by this method may provide clues for additional media optimization process.

It is assumed that transfectomas that are selected based on expression level of one or more of these cellular proteins will retain their respective phenotype when they are cultivated in bioreactors. If implemented, a screening protocol comprising of 1) H-ch and L-ch mRNA copy number for optimal expression and stability, 2) a QPCR analysis of transcripts of cellular proteins for desirable product characteristics, and 3) analysis of intra-cellular Ab content by flow cytometry for selecting stable cell lines is envisioned. Additionally, a screening step comprising of markers identified in the current proteomic study can be incorporated, as these markers are indicative of high productivity in fed batch bioreactors. Not unexpectedly, the number of clones that would be screened at successive rounds will decrease. In instances where expression of specific cellular proteins have an effect on productivity, transfection of host cell lines can be engineered for superior productivity via appropriate modulation of these proteins. Moreover, it is possible that when used in conjunction with methods that boost transcription of H-ch and L-ch genes [14], we can develop cell lines with very high productivity.

Supplementary file information

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References

1. Buss NA, Henderson SJ, McFarlane M, Shenton JM, de Haan L (2012) Monoclonal antibody therapeutics: history and future. Curr Opin Pharmacol 12: 615-622.
2. Birch JR, Racher AJ (2006) Antibody production. Adv Drug Deliv Rev 58: 671-685.
3. Tigges M, Fussenegger M (2006) Xbp1-based engineering of secretory capacity enhances the productivity of Chinese hamster ovary cells. Metab Eng 8: 264-272.
4. Le H, Castro-Melchor M, Hakemeyer C, Jung C, Szperalski B, et al. (2011) Discerning key parameters influencing high productivity and quality through recognition of patterns in process data. BMC Proc 5: P91.
5. Mulukutla BC, Khan S, Lange A, Hu WS (2010) Glucose metabolism in mammalian cell culture: new insights for tweaking vintage pathways. Trends Biotechnol 28: 476-484.
6. Dean J, Reddy P (2013) Metabolic analysis of antibody producing CHO cells in fed-batch production. Biotechnol Bioeng 110: 1735-1747.
7. Templeton N, Dean J, Reddy P, Young JD (2013) Peak antibody production is associated with increased oxidative metabolism in an industrially relevant fed-batch CHO cell culture. Biotechnol Bioeng.
8. deZengotita VM, Miller WM, Aunins JG, Zhou W (2000) Phosphate feeding improves high-cell-concentration NS0 myeloma culture performance for monoclonal antibody production. Biotechnol Bioeng 69: 566-576.
9. Suzuki E, Ollis DF (1990) Enhanced antibody production at slowed growth rates: experimental demonstration and a simple structured model. Biotechnol Prog 6: 231-236.
10. Gagnon M, Hiller G, Luan YT, Kittredge A, DeFelice J, et al. (2011) High-end pH-controlled delivery of glucose effectively suppresses lactate accumulation in CHO fed-batch cultures. Biotechnol Bioeng 108: 1328-1337.
11. Fussenegger M, Schlatter S, Dätwyler D, Mazur X, Bailey JE (1998) Controlled proliferation by multigene metabolic engineering enhances the productivity of Chinese hamster ovary cells. Nat Biotechnol 16: 468-472.
12. Dreesen IA, Fussenegger M (2011) Ectopic expression of human mTOR increases viability, robustness, cell size, proliferation, and antibody production of chinese hamster ovary cells. Biotechnol Bioeng 108: 853-866.
13. Meents H, Enenkel B, Eppenberger HM, Werner RG, Fussenegger M (2002) Impact of coexpression and coamplification of sICAM and antiapoptosis determinants bcl-2/bcl-x(L) on productivity, cell survival, and mitochondria number in CHO-DG44 grown in suspension and serum-free media. Biotechnol Bioeng 80: 706-716.
14. Böhm E, Voglauer R, Steinfellner W, Kunert R, Borth N, et al. (2004) Screening for improved cell performance: selection of subclones with altered production kinetics or improved stability by cell sorting. Biotechnol Bioeng 88: 699-706.
15. Zustiak MP, Dorai H, Betenbaugh MJ, Sauerwald TM (2012) Controlling apoptosis to optimize yields of proteins from mammalian cells. Methods Mol Biol 801: 111-123.
16. Dorai H, Corisdeo S, Ellis D, Kinney C, Chomo M, et al. (2012) Early prediction of instability of Chinese hamster ovary cell lines expressing recombinant antibodies and antibody-fusion proteins. Biotechnol Bioeng 109: 1016-1030.
17. Dorai H, Csirke B, Scallon B, Ganguly S (2006) Correlation of heavy and light chain mRNA copy numbers to antibody productivity in mouse myeloma production cell lines. Hybridoma (Larchmt) 25: 1-9.
18. Barnes LM, Bentley CM, Dickson AJ (2001) Characterization of the stability of recombinant protein production in the GS-NS0 expression system. Biotechnol Bioeng 73: 261-270.

19. Barnes LM, Bentley CM, Dickson AJ (2003) Stability of protein production from recombinant mammalian cells. Biotechnol Bioeng 81: 631-639.

20. Barnes LM, Bentley CM, Dickson AJ (2003) Stability of recombinant protein production in the GS-NS0 expression system is unaffected by cryopreservation. Biotechnol Prog 19: 233-237.

21. Arden N, Nivitchanyong T, Betenbaugh MJ (2004) Cell engineering blocks cell stress and improves biotherapeutic production. BioProcess J 3: 23-28.

22. Sauerwald TM, Betenbaugh MJ (2002) Apoptosis in Biotechnology: Its role in mammalian cell culture and methods of inhibition. BioProcess J 1: 61-69.

23. Dorai H, Ellis D, Keung YS, Campbell M, Zhuang M, et al. (2010) Combining high-throughput screening of caspase activity with anti-apoptosis genes for development of robust CHO production cell lines. Biotechnol Prog 26: 1367-1381.

24. Dorai H, Kyung YS, Ellis D, Kinney C, Lin C, et al. (2009) Expression of anti-apoptosis genes alters lactate metabolism of Chinese Hamster Ovary cells in culture. Biotechnol Bioeng 103: 592-608.

25. Mazur X, Fussenegger M, Renner WA, Bailey JE (1998) Higher productivity of growth-arrested Chinese hamster ovary cells expressing the cyclin-dependent kinase inhibitor p27. Biotechnol Prog 14: 705-713.

26. Dorai H, Santiago A, Campbell M, Tang QM, Lewis MJ, et al. (2011) Characterization of the proteases involved in the N-terminal clipping of glucagon-like-peptide-1-antibody fusion proteins. Biotechnol Prog 27: 220-231.

27. Dorai H, Li K, Huang CC, Bittner A, Galindo J, et al. (2007) Genome-wide analysis of mouse myeloma cell lines expressing therapeutic antibodies. Biotechnol Prog 23: 911-920.

28. Yee JC, de Leon Gatti M, Philp RJ, Yap M, Hu WS (2008) Genomic and proteomic exploration of CHO and hybridoma cells under sodium butyrate treatment. Biotechnol Bioeng 99: 1186-1204.

29. Datta P, Linhardt RJ, Sharfstein ST (2013) An 'omics approach towards CHO cell engineering. Biotechnol Bioeng 110: 1255-1271.

30. Selvarasu S, Ho YS, Chong WP, Wong NS, Yusufi FN, et al. (2012) Combined *in silico* modeling and metabolomics analysis to characterize fed-batch CHO cell culture. Biotechnol Bioeng 109: 1415-1429.

31. Baycin-Hizal D, Tabb DL, Chaerkady R, Chen L, Lewis NE, et al. (2012) Proteomic analysis of Chinese hamster ovary cells. J Proteome Res 11: 5265-5276.

32. Baik JY, Lee MS, An SR, Yoon SK, Joo EJ, et al. (2006) Initial transcriptome and proteome analyses of low culture temperature-induced expression in CHO cells producing erythropoietin. Biotechnol Bioeng 93: 361-371.

33. Kim JY, Kim YG, Lee GM (2012) Differential in-gel electrophoresis (DIGE) analysis of CHO cells under hyperosmotic pressure: osmoprotective effect of glycine betaine addition. Biotechnol Bioeng 109: 1395-1403.

34. Meleady P, Hoffrogge R, Henry M, Rupp O, Bort JH, et al. (2012) Utilization and evaluation of CHO-specific sequence databases for mass spectrometry based proteomics. Biotechnol Bioeng 109: 1386-1394.

35. Hammond S, Kaplarevic M, Borth N, Betenbaugh MJ, Lee KH (2012) Chinese hamster genome database: an online resource for the CHO community at www.CHOgenome.org. Biotechnol Bioeng 109: 1353-1356.

36. Xu X, Nagarajan H, Lewis NE, Pan S, Cai Z, et al. (2011) The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. Nat Biotechnol 29: 735-741.

37. Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, et al. (1999) Housekeeping genes as internal standards: use and limits. J Biotechnol 75: 291-295.

38. Anderson L, Hunter CL (2006) Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. Mol Cell Proteomics 5: 573-588.

39. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, et al. (2005) Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics 4: 1265-1272.

40. Schlatter S, Stansfield SH, Dinnis DM, Racher AJ, Birch JR, et al. (2005) On the optimal ratio of heavy to light chain genes for efficient recombinant antibody production by CHO cells. Biotechnol Prog 21: 122-133.

41. Porter AJ, Dickson AJ, Racher AJ (2010) Strategies for selecting recombinant CHO cell lines for cGMP manufacturing: realizing the potential in bioreactors. Biotechnol Prog 26: 1446-1454.

42. Porter AJ, Mohindra A, Porter JM, Racher AJ (2011) Does earlier use of productivity enhancers during cell line selection lead to the identification of more productive cell lines? BMC Proc 5: P9.

43. Kaufmann H, Mazur X, Fussenegger M, Bailey JE (1999) Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells. Biotechnol Bioeng 63: 573-582.

44. Elias DA, Tollaksen SL, Kennedy DW, Mottaz HM, Giometti CS, et al. (2008) The influence of cultivation methods on *Shewanella oneidensis* physiology and proteome expression. Arch Microbiol 189: 313-324.