COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

Metabolite Profiling of Recombinant CHO Cells: Designing Tailored Feeding Regimes That Enhance Recombinant Antibody Production

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ABSTRACT: Chinese hamster ovary (CHO) cells are the primary platform for commercial expression of recombinant therapeutic proteins. Obtaining maximum production from the expression platform requires optimal cell culture medium (and associated nutrient feeds). We have used metabolite profiling to define the balance of intracellular and extracellular metabolites during the production process of a CHO cell line expressing a recombinant IgG4 antibody. Using this metabolite profiling approach, it was possible to identify nutrient limitations, which acted as bottlenecks for antibody production, and subsequently develop a simple feeding regime to relieve these metabolic bottlenecks. This metabolite profiling-based strategy was used to design a targeted, low cost nutrient feed that increased cell biomass by 35% and doubled the antibody titer. This approach, with the potential for utilization in non-specialized laboratories, can be applied universally to the optimization of production of commercially important biopharmaceuticals.

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KEYWORDS: metabolite profiling; antibody; glutamine synthetase; CHO cells; bioprocessing; GC-MS

Abbreviations: CHO, Chinese hamster ovary; GC-MS, gas chromatography-mass spectrometry.

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Matching mammalian host cells with the appropriate culture environment is a multi-factorial challenge that has acute commercial significance for production of biopharmaceuticals at industrial scale. At present, the design of culture media and feeding regimes for antibody production processes is based largely on "design-of-experiment" approaches (Castro et al., 1992; Kim and Lee, 2009; Lee et al., 1999). In this study, we demonstrate an alternative metabolite profiling-based strategy towards rationalization of medium design which can be universally applied to any biopharmaceutical process. Net intra- and extracellular changes in nutrient and metabolite concentrations were measured during batch culture of a recombinant CHO cell line. This type of approach can be performed with extracellular data alone; however, the additional data generated through parallel intracellular sampling allows a deeper understanding of the integrated cellular consequences of changes to external nutrients. This includes understanding bottlenecks in the transport of nutrients into and out of the cells that could lead to nutrient limitations or build-up of by-products inside the cell that significantly impact growth and productivity. The CHO cells used in this study were engineered to produce an IgG4 antibody in the glutamine synthetase (GS) expression system, and were

grown in bioreactors using a proprietary defined medium. Extracellular (footprint) samples were generated by direct sampling of the medium. The intracellular (fingerprint) samples were generated using methods developed previously (Sellick et al., 2009a, 2010). The metabolic profiles generated by gas chromatography-mass spectrometry (GC-MS) were used to identify key limiting nutrients. This information was then applied to design a simple inexpensive nutrient feed that increased cell biomass by 35% and the overall antibody yield by more than 100% compared with basal medium.

Footprint and fingerprint profiles were generated throughout culture and metabolite profiles for CHO cells were mapped onto metabolic pathways. For clarity, Figure 1 details a summation of multiple determinations of a subset of the key intracellular and extracellular metabolites. The complete dataset is included in the Supplemental data (Suppl. Fig. 1). Whilst replicate experiments were undertaken (in terms of analysis and timepoints), we have selected to display the data in the summation format of Figure 1, as we believe that the visual simplicity (combining parallel intra- and extracellullar metabolites) eases interpretation of complex datasets. Footprint data showed usage of glucose, pyruvate and all detected amino acids except glycine, and, to a lesser extent, alanine, both of which accumulated during culture. Other metabolites that accumulated in the medium were lactate, glycerol, and sorbitol. These results are consistent with previously reported data (Dorai et al., 2009; Ma et al., 2009; Rose, 2009). Mapping the exhaustion and the start point for the accumulation of metabolites onto a timeline (Fig. 2A) demonstrated that changes in the medium metabolite profile clearly coincided with changes in the growth phases of the cells. The transition from exponential to stationary phase was marked by the exhaustion of asparagine, aspartate, glutamate, and pyruvate, and, as the cells fully entered stationary phase, glycerol and sorbitol accumulation was observed in the medium. Finally, the transition from stationary to decline phases was marked by the exhaustion of glucose, leucine, lysine, and serine.

The intracellular metabolites (fingerprint) were also measured using rapid harvesting based on quenching and extraction methods that we developed, which minimize contamination from extracellular metabolites, metabolite turnover, and decomposition of labile metabolites (Sellick et al., 2009a, 2010). Fingerprint data obtained from parallel sampling of intracellular metabolites correlated with the footprint patterns. Analysis of data from intracellular sampling demonstrated that metabolites involved in glycolysis and the TCA cycle were present inside the cells during exponential growth but were absent or significantly decreased during decline phase. Lactate amounts were also linked to the growth phase. The decline in intracellular amounts of these metabolites correlated with the transition from exponential growth to stationary and decline phases. This transition between exponential growth and stationary phase was also marked by the intracellular appearance of glycerol-3-phosphate, glycerol (presumably synthesized from glycerol-3-phosphate), and sorbitol, metabolites that appeared in cells in advance of accumulation in medium. Our interpretation of all data from footprint and fingerprint profiling during batch culture is that the entry of cells into stationary phase is accompanied by a switch in metabolism with glucose carbon skeletons being redirected from (or unable to enter) the TCA cycle. As a consequence glucose carbon skeletons are redirected to alternative metabolic endpoints, as seen with glycerol and sorbitol (which may also be formed as osmolytes; Yancey, 2005). Our hypothesis is that this represents an adaptation to lack of TCA cycle intermediates (associated, in the GS-CHO cell system, with depletion of aspartate, asparagine, and glutamate; Figs. 1 and 2A) and that the redirection of glucose (and potentially other metabolites) permits ATP generation and maintenance of cellular redox state (permitting NADH and NADPH reoxidation—illustrated by decreased lactate and accumulation of glycerol and sorbitol). We would propose that changes to the metabolic profile presents a "crisis-management" situation for cells, supporting maintenance but limiting growth and formation of new macromolecular components. Intracellular amino acid content of the cells generally decreased during culture and reflected changes observed in the extracellular medium. The most significant of these changes were observed for aspartate, asparagine, and glutamate, which fell to below detection levels by GC-MS once the cells entered stationary phase (Figs. 1 and 2A).

The entry of cells into decline phase of batch culture was associated with further decreases to the relative concentrations of the intermediates of glycolysis and the TCA cycle. This may reflect the depletion of exogenous glucose when the cells entered the decline phase, which would result in general starvation and depletion of central metabolic intermediates. Lactate was produced inside the cells during exponential growth and the stationary phase, but the relative concentration fell as the cells entered the decline phase.

The link between depletion of aspartate, asparagine, glutamate, and pyruvate at the beginning of the stationary phase suggested that these amino acids and pyruvate were growth-limiting nutrients. Therefore, we examined the effect of adding feeds containing these nutrients on cell growth and antibody expression (Fig. 2B and C). In this proof-of-principle experiment each of the nutrients were re-fed back to the concentration determined for the initial medium. Supplementation with the aspartate, asparagine, glutamate, and pyruvate feed maintained exponential growth for an extra day, confirming that these nutrients were indeed growth limiting. This resulted in an increased maximum viable cell density (+35%). The integral viable cell numbers (IVC; the accumulated cell numbers, a measure of cumulative cell growth) increased to $26.8 \pm 1.29 \times 10^6$ cells/day/mL compared with $23.8 \pm 1.38 \times 10^6$ cells/day/mL in the control cultures. More importantly, the antibody titer increased by 75%, demonstrating that these nutrients were also limiting for antibody production. However, the cells died more quickly, compared to the unfed controls. This was caused by the depletion of many of the nutrients (glucose,

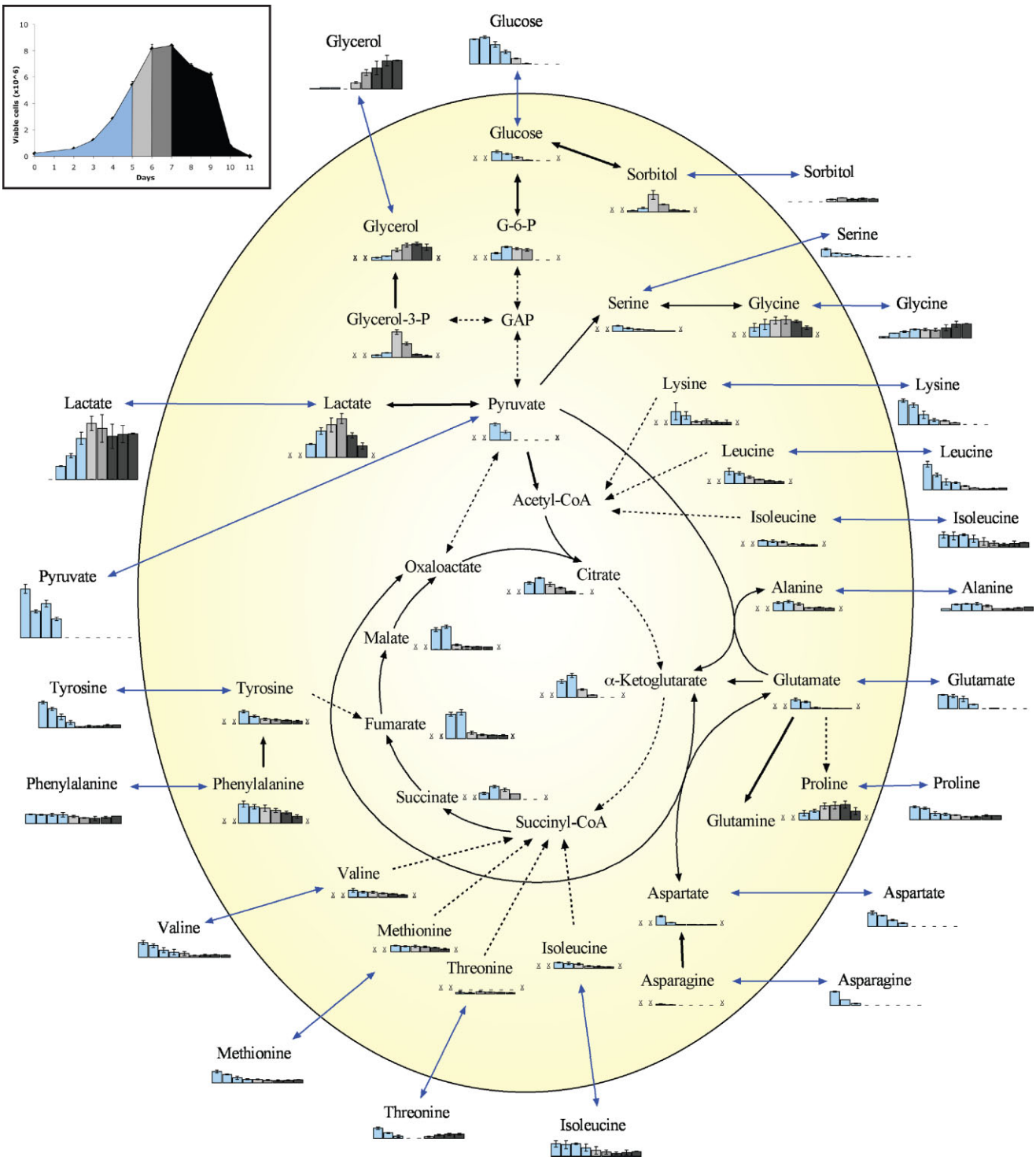

Figure 1. Central carbon metabolism of CHO cells and the interaction with the extracellular medium. Fingerprint and footprint samples from the bioreactor were analyzed and the bar charts represent the quantities of metabolites on days 0, 3, 4, 5, 6, 7, 8, 9, and 11 (fingerprint samples were not harvested on days 0, 3, and 11). For each metabolite, days on which the metabolite was not detected (—) or when the cell density was too low to take samples (x) are indicated. The bars are colored to indicate the stage of growth: blue = exponential; light grey = stationary; dark grey = decline (as indicated by the insert growth profile). Values represent arbitrary units normalized to an internal standard and standard deviation error bars are shown. The bar charts are mapped onto the central metabolic pathways, with single reactions indicated with solid black lines and multiple steps by dashed lines. Blue lines indicate transport or diffusion across the cell membrane. Arrows indicate the direction of reactions/transport with double arrowheads indicating that the reaction/transport is reversible.

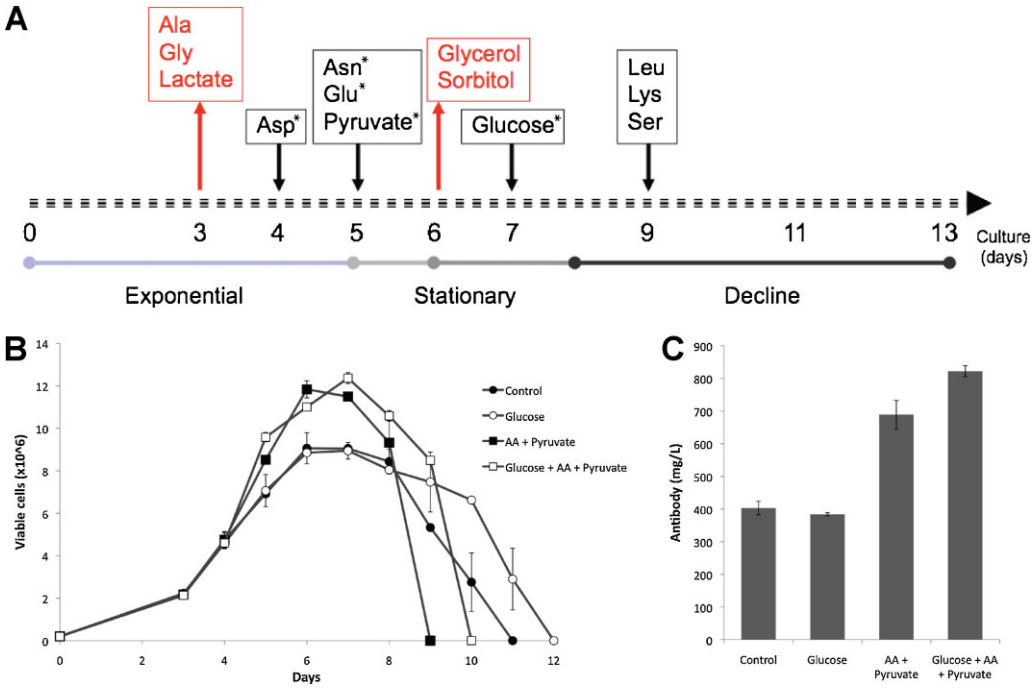

Figure 2. Feed addition based on metabolite profiling of the CHO cells. **A**: Timeline showing when key nutrients or metabolites were depleted (black arrows) or started to accumulate (red arrows) in the growth medium in the bioreactor (Fig. 1). **B**,**C**: Feeds containing nutrients that had been depleted (* in A) were added to cultures on day 4. The feeds contained glucose, an amino acid mixture (asparagine, aspartate, and glutamate; AA) with pyruvate, or AA with pyruvate and glucose together. Cell growth (B) and maximum antibody titer (C) were measured and compared to an unfed control. Maximum antibody titers were achieved on day 8 for all cultures. Values represent the average of four biological replicates, and standard error of the mean (SEM) error bars are shown.

pyruvate, and several amino acids) in the medium by day 8 as a result of the increased growth and antibody production (data not shown).

Since entry into the decline phase of cell culture was associated with depletion of glucose (Fig. 2A), we tested the effect of feeding glucose (alone or added to other feed components). Addition of glucose alone had no effect on the growth rate of cells, maximum viable cell numbers, antibody titer, or IVC ($23.4 \pm 1.22 \times 10^6$ cells/day/mL compared to $23.8 \pm 1.38 \times 10^6$ cells/day/mL for the control, on day 8). Therefore, glucose was not growth limiting but did prolong the stationary phase, and delayed the start of the decline phase. When glucose was added in combination with aspartate, asparagine, glutamate, and pyruvate, as before, the additional glucose had no further effect on growth or maximum cell numbers (IVC was $27.1 \pm 0.89 \times 10^6$ cells/day/mL in the presence of glucose compared with $26.8 \pm 1.29 \times 10^6$ cells/day/mL in its absence). However, once again glucose extended the stationary phase (by 1 day). Most importantly, the antibody titer increased by 100% compared with the unfed control, an additional increase of 25% compared with the cultures fed with amino acids and pyruvate alone.

These studies demonstrated that metabolite profiling could be used to identify nutrients that were limiting for growth and antibody production, and for cell survival. This made it possible to design feeds containing the limiting nutrients and use them to double the production of a monoclonal antibody. Critically, the approach outlined facilitates fundamental understanding of mechanisms of action and offers the potential to engineer cells towards an improved cell phenotype that reflects a "good" metabolism for IVC and specific productivity. Consequently, a final stage in this investigation was to confirm that the nutrient supplementation was linked to changes in metabolism within the cells. In experienced hands this is a relatively straightforward task and would allow specific feed development that would match to individual clonal variants, observed even when a platform technology is used. We therefore performed footprint analysis of the medium from the rational feed experiments (Fig. 3) to allow the "cause and effect" of metabolite addition and usage to be determined. Analysis of components included in the rational feeds (Fig. 3; marked with *) showed that increased amounts of each metabolite were present the day after feed addition (day 5; except for asparagine which was consumed within the first 24 h after addition). High asparagine consumption is a feature noted by others using the GS-CHO expression system (Dorai et al., 2009; Ma et al., 2009) and is likely to link to the unique dual role of asparagine as a nitrogen donor and as a feed for replenishment of TCA cycle intermediates. Although addition of glucose alone (in the

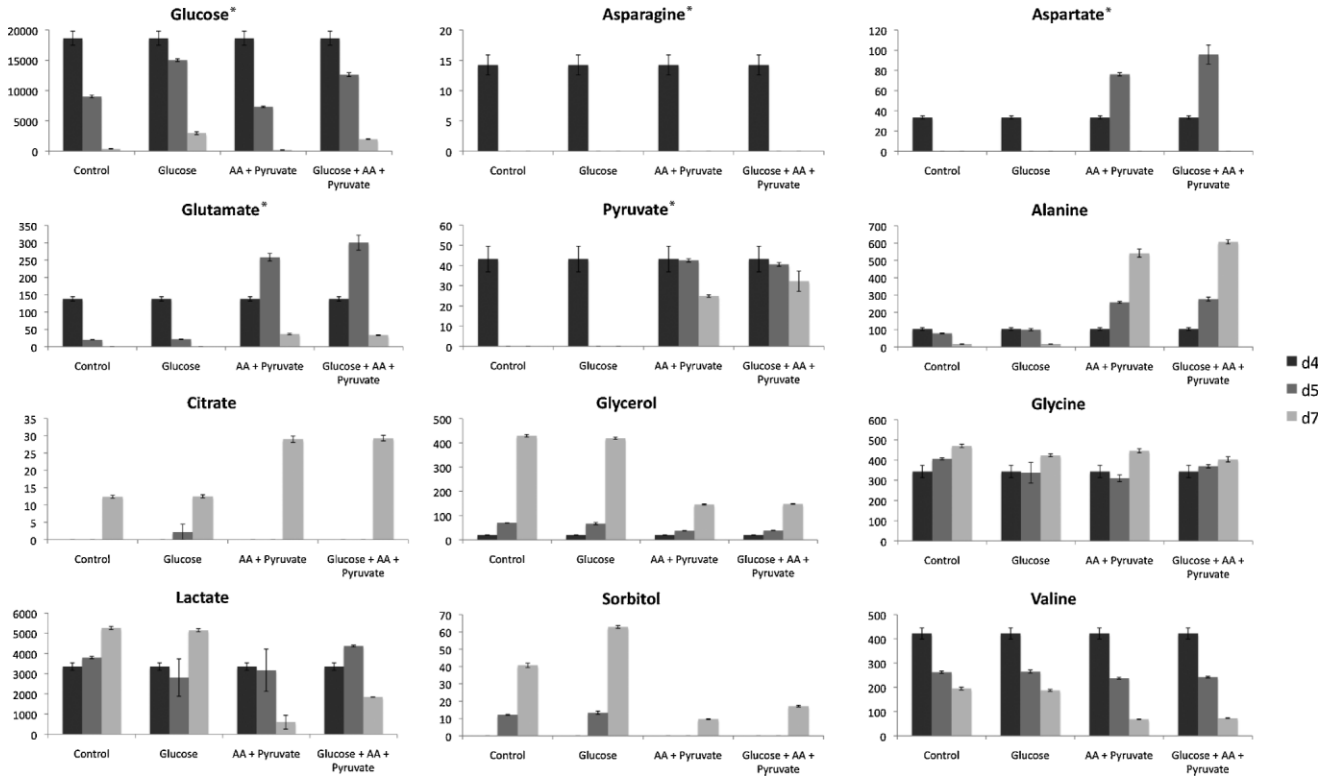

Figure 3. Effects of feed addition on medium component usage. Medium from the cultures in Figure 2B and C was subjected to GC-MS analysis. Medium samples from days 4 (before feed addition; black), 5 (dark grey), and 7 (light grey) were analyzed. Metabolites that were included in the feeds are indicated (*). Values are arbitrary units normalized to an internal standard and represent the average of four biological replicates, with standard error of the mean (SEM) error bars.

absence of the other four feed components) extended cell culture time by approximately 24 h (Fig. 2B), this intervention did not have a significant effect on the usage of medium metabolites or the excretion of metabolites from the cells (Fig. 3). By contrast, there were significant changes in the footprint of cultures supplemented with the aspartate, asparagine, glutamate, and pyruvate feed. Under those conditions much less lactate, glycerol, and sorbitol were produced, but this was accompanied by a significant increase in citrate and alanine production, features that are consistent with an increased flow of metabolites through glycolysis and into the TCA cycle and providing support for enhanced oxidative phosphorylation.

These data address how the efficiency of the TCA cycle may directly influence cell growth and support of recombinant gene product synthesis. Others have suggested that transformed mammalian cells have impaired TCA cycle function, with limited capacity to perform the steps that lead to production of α-ketoglutarate from citrate (Baggetto et al., 1992; Sengupta et al., 2010). This "truncation" of the TCA cycle has been used to explain the release of citrate (as an "overflow") from cultured CHO cells (Ma et al., 2009; a feature characterized in our present study) and this presents a limitation to the potential optimization of usage of components in culture media. Our initial metabolite profiling identified the significance of glutamate and pyruvate for maintenance of cell growth and recombinant protein production. Addition of these two components as part of a feed permits the continued synthesis of α-ketoglutarate directly (via the activity of glutamate dehydrogenase) and indirectly (via the activity of alanine aminotransferase, with concomitant production of alanine) hence permitting an enhanced TCA activity (bypassing any potential truncation). Feeds containing amino acids and pyruvate decreased medium lactate amounts (Fig. 3). This observation correlates with the comments made by Ma et al. (2009), who have referenced a series of publications which have shown that decreased production of lactate is linked to increased maximum cell density and increased recombinant protein yield. Decreased lactate amounts would not intuitively be expected to follow from addition of feeds containing pyruvate (which might be expected to increase lactate) but our working hypothesis is that the greater efficiency of the TCA cycle permits more efficient utilization of the available nutrients.

In keeping with this hypothesis and the observed increases in IVCs and recombinant antibody titers, amino acid usage was increased in the fed cultures. For example, the relative valine concentration after day 7 was 50% of that in the unfed

cultures that were not supplemented with the amino acid/pyruvate feed. Similarly, a group of essential amino acids (leucine, lysine, and tryptophan) were completely exhausted by day 7 following feed addition (data not shown). This would explain the dramatic entry of cells into decline phase. Therefore, a key feature illuminated by our studies is that re-iterative analysis of limiting metabolites will illuminate further sequential refinement of the nutrient feed to overcome these new limitations.

The work presented here clearly demonstrates the power of metabolite profiling (and associated interpretations) for rapid experimental design of simple (and potentially clone-specific) feeds for mammalian (or other host) cell bioprocesses. A further consideration in this type of rationally feed design is that changes in culture conditions can have a significant impact on product quality (Schiestl et al., 2011). Therefore, in any studies directed to a therapeutic perspective, it would be essential that the effects on product quality of changing feeding regimes are considered and appropriately monitored to ensure desirable and consistent product characteristics. The ability to design culture media and nutrient feeds rationally has direct commercial significance for improving antibody titers in industrial manufacturing processes. Therefore, the metabolite profiling process described here represents an important new tool in the optimization of biopharmaceutical production.

Methods

Media and Reagents

CD-OptiCHO medium was obtained from Invitrogen (Carlsbad, CA); the chemical composition is defined but confidential. All other reagents were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise stated.

Cell Lines, Cell Culture, and Growth Assessment

The GS-CHO cell line LB01 was obtained from Lonza Biologics (Slough, UK), and was generated by transfection of CHOK1SV cells with a construct containing GS and a chimeric IgG4 antibody (cB72.3). Stocks of LB01 cells were revived in 20 mL CD-OptiCHO medium (Invitrogen), supplemented with 25 μM methionine sulfoximine (MSX). The cells were sub-cultured every 3–4 days with a seeding density of 0.2×10^6 cells/mL and were grown in 250 mL Erlenmeyer flasks in a volume of 50 mL medium. All cultures were grown at 37°C with 100 rpm orbital speed. Bioreactors were run according to the method described by Sellick et al. (2009b). Feeds were added on day 4 of culture from a 100× stock to a final concentration of 0.2 g/L. Growth was assessed by light microscopy using an improved Neubauer haemocytometer at 24-h intervals. Samples were appropriately diluted and mixed 1:1 with 0.5% Trypan blue in phosphate buffered saline (PBS).

Sampling for Footprint Analysis

Clarified medium samples (20 μL) were mixed with 200 μL methanol, vortexed briefly, and centrifuged at 15,000*g* for 5 min. The supernatant was removed and lyophilized.

Sampling for Fingerprint Analysis

The cells were rapidly quenched by addition of 1×10^7 cells to 5 volumes 60% methanol supplemented with 0.85% (w/v) ammonium bicarbonate (AMBIC, pH 7.4) at −40°C as described by Sellick et al. (2009a). The metabolites were extracted from the cells using two 100% methanol extractions followed by one water extraction as described by Sellick et al. (2010).

Sample Derivatization and GC-MS Analysis

Footprint and fingerprint samples were analyzed by GC-MS as described by Sellick et al. (2010).

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