Research Article

Metabolite profiling of CHO cells: Molecular reflections of bioprocessing effectiveness

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Abbreviations: **CHO**, Chinese Hamster Ovary; **GC-MS**, Gas chromatography-mass spectrometry

Abstract

Whilst development of medium and feeds has provided major advances in recombinant protein production in CHO cell, the fundamental understanding is limited. We have applied metabolite profiling with established robust (GC-MS) analytics to define the molecular loci by which two yield-enhancing feeds improve recombinant antibody yields from a model GS-CHO cell line. With data across core metabolic pathways, that report on metabolism within several cellular compartments, these data identify key metabolites and events associated with increased cell survival and specific productivity of cells. Of particular importance, increased process efficiency was linked to the functional activity of the mitochondria, with the amount and time course of use/production of intermediates of the citric acid cycle, for uses such as lipid biosynthesis, precursor generation and energy production, providing direct indicators of cellular status with respect to productivity. The data provide clear association between specific cellular metabolic indicators and cell process efficiency, extending from prior indications of the relevance of lactate metabolic balance to other redox sinks (glycerol, sorbitol and threitol). The information, and its interpretation, identifies targets for engineering cell culture efficiency, either from genetic or environmental perspectives, and greater understanding of the significance of specific medium components towards overall CHO cell bioprocessing.

1 Introduction

Biopharmaceuticals (*e.g.* antibodies) by their nature are complex molecules requiring specific post-translational modifications for activity [1]. It is for this reason that these proteins are produced in mammalian expression platforms that can correctly perform the required modifications [2]. The current workhorses of the biotechnology industry are various lineages of Chinese hamster ovary (CHO) cells [3]. These cells are able to correctly modify the proteins and, with suitable process development, produce final titres measured in multiple grams per litre [4]. The aim of any production process is to manufacture as much product (of appropriate quality) as possible in the shortest time to maximise efficiency.

To achieve high titres the cells require a base medium to support the early stages of culture and, to extend the period of productivity, subsequent nutrient feeds to replace limiting nutrients [5]. Together the combination of the medium and the feeding regime should provide the optimum conditions for maximising product production. We previously demonstrated the power of metabolite profiling for the rapid design of simple feeds that addressed the specific needs of cells during culture [6]. By replacing just the key limiting nutrients (glucose, asparagine, aspartate, glutamate and pyruvate) it was possible to increase maximum cell numbers (35%), the integral viable cell numbers (IVC; 14%) and, most importantly, the antibody titre (100%). However, proprietary industrial feeds are routinely highly complex containing many different components at high concentrations. The addition of these concentrated feeds has an impact on ratio of the nutrients available to the cells and increases the osmolality of the culture [7].

Therefore, it is important to understand the molecular rationale by which feeds favor greatest efficiency of cellular metabolism and, indeed, to define metabolic profiles associated with desirable production phenotypes (an optimal combination of high biomass, high specific productivity and product exhibiting appropriate quality attributes). Such understanding will enable design of new generation feeds to match purpose or cell intervention approaches to select/engineer novel cellular platforms and culture processes.

To date, development of improved media and feeds has largely occurred via empirical “brute force” screening with relatively limited precise understanding of the metabolism of CHO cells that underpins effectiveness of cell growth or recombinant protein production [8]. Very early studies indicated a generalised reliance on glycolysis and glutaminolysis for energy production in early cell growth and information was available on other selected aspects of metabolism [9, 10]. However, recently, we have seen the optimisation of analytical technologies that facilitate simultaneous characterisation of multiple extracellular and intracellular metabolites for CHO cells in varied culture and environmental conditions [6, 11-15]. The mining and analysis of complex datasets from metabolite profiling (metabolomics approaches) has facilitated a greater understanding of the relationships between cells, medium, feeds and functional product generation [6, 15-17]. The information is also being used to build verifiable models of CHO cell metabolism and in turn such models will have the predictive potential towards the design of enhanced cell line culture processes. [18-20]

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It was with this background in mind that we set out to develop an understanding of the metabolic events that underpinned the ability of feeds to enhance the growth, survival and productivity of a model CHO cell line in a commercial (and widely used) medium (CD-OptiCHO). Using both intracellular and extracellular metabolite profiling, we have examined the metabolic consequences of addition of two commercial feeds on cellular performance and have shown that key metabolic stages (in relation to cellular redox handling and integration between cytosolic and mitochondrial metabolism) are influenced by feeds. These data highlight limiting steps that offer the potential for process enhancement.

2 Materials and methods

2.1 Media and Reagents

CD-OptiCHO medium and CHO CD EfficientFeed A and B were obtained from Invitrogen (Carlsbad, US). The chemical composition is defined but confidential. All other reagents were obtained from Sigma-Aldrich unless otherwise stated.

2.2 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 glutamine synthetase (GS) and a chimeric IgG4 antibody (cB72.3) [6]. Stocks of LB01 cells were revived in 20 ml CD-OptiCHO medium (Invitrogen), supplemented with 25 μ M methionine sulfoximine (MSX). The cells were sub-cultured every 3-4 days with a seeding density of 0.2×10^6 cells ml⁻¹ 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 shaking. Bioreactors were run according to the method described previously [21]. Where appropriate, feeds were added at 30% (v/v) culture volume at the start of culture. The starting volumes for cultures with feed addition were decreased such that the final volume after feed addition was equal to the unfed cultures. 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 PBS.

2.3 Sampling for footprint analysis

Clarified medium samples (20 μ l) were mixed with 200 μ l methanol, vortexed briefly and centrifuged at 15000 $\times g$ for 5 min. The supernatant was removed and lyophilised.

2.4 Sampling for fingerprint analysis

Samples for assessment of intracellular metabolites were harvested from day 4 of culture onwards (due to relatively low cell densities prior to this stage and limited culture volume for sampling). 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 [22]. Addition of the cells to the quenching solution increased the temperature by no more than 15°C . The cells in the quenching solution were then centrifuged at 1000 $\times g$ for 1 min and the quenching solution was removed [22]. The metabolites were extracted from cells using two 100% methanol extractions followed by a single water extraction. The cell pellet was resuspended in 0.5 ml 100% methanol followed by flash freezing in liquid nitrogen. After thawing on ice at 4°C , samples were vortexed for 30 s, centrifuged at 800 $\times g$ and the supernatant removed. The pellet was resuspended in a further 0.5 ml of the methanol and the extraction repeated. The cell pellet was resuspended in 0.3 ml ice-cold milliQ water followed by flash freezing in liquid nitrogen. After thawing on ice at 4°C samples were vortexed for 30 s, centrifuged at 15000 $\times g$ and the supernatant removed. The methanol and water extracts were pooled, centrifuged at 15000 $\times g$ for 1 min and the supernatant removed and lyophilized [23].

2.5 Sample derivatization and GC-MS analysis

Footprint and fingerprint samples were analysed by GC-MS as described earlier [23]. Briefly, lyophilised intracellular metabolite extracts were prepared for GC-MS analysis by a two-stage derivatisation procedure (methoxyamination followed by trimethylsilylation). GC-MS analysis of the derivatised samples was performed on a 7890A GC System (Agilent Technologies), fitted with a DB-5MS+DG column (Agilent Technologies; 250 μ m x 30 m, 0.25 μ m film thickness with 10 m duraguard), coupled to a 5975C Inert XL MSD with Triple-Axis Detector (Agilent Technologies) using the manufacturers software (MSD ChemStation). Metabolite peaks in the raw chromatograms were identified using MSD ChemStation (Agilent Technologies) to search custom libraries based on the Agilent Fiehn GC/MS Metabolomics RTL Library (<http://fiehnlab.ucdavis.edu/Metabolite-Library-2007>). Metabolite identifications were based on retention times and fragmentation patterns. The data were combined using an in-house Microsoft Excel (Microsoft Corporation) macro and normalized to the retention time locking (RTL) compound (myristic acid d27).

3 Results

3.1 Characterisation of cell growth and antibody production in batch and fed-batch culture

Preliminary results with addition of CHO CD EfficientFeeds $\square$ to different volume:volume percentages and at a series of different time intervals, indicated that the addition of 30% (v/v) of either CHO CD EfficientFeed $\square$ A or B at the start of batch culture generated the greatest consequence for cell growth and

production. In the absence of feeding (i.e. in OptiCHO medium alone), cells in the bioreactor exhibited a short lag period before entering an exponential phase, reaching a density of just under 9×10^6 cells/ml before entering decline phase with a minimal stationary phase (Figure 1A). The inclusion of CHO CD EfficientFeeds A or B produced little change to cell growth rate during exponential phase (days 0-4; specific growth rate; Unfed, 0.72 d^{-1} , EfficientFeed A, 0.75 d^{-1} , EfficientFeedB, 0.79 d^{-1}) but generated increased maximum cell densities and an extension to the period before cells entered decline phase. The feeds each produced slightly different growth profiles, with CHO CD EfficientFeed B achieving a slightly lower maximum cell density but showing more of an extended stationary phase but both generated more than twice the total number of cells across the entire functional batch culture period (for total culture period; cumulative cell number; Unfed, 40.84×10^6 cells. ml^{-1}d , EfficientFeed A, 81.17×10^6 cells. ml^{-1}d , EfficientFeedB, 90.19×10^6 cells. ml^{-1}d). Viability was retained for longer in the presence of each feed (Data not shown) and when cells entered decline phase the rate of cell death was similar in the presence and absence of feed.

In the absence of feeds, antibody production showed greatest increase as cells reached the end of the exponential growth phase and maximum yield was $0.35\text{--}0.40 \text{ g.L}^{-1}$ by 10-12 days of batch culture (Figure 1B). Although the pattern of growth differed between CHO CD EfficientFeeds A and B, addition of either feed produced a similar stimulation of antibody production which was noted from day 5 of culture onwards, a period when the consequence of feed addition on cell growth and maintenance had been noted. As a consequence of retained cell

biomass, both feeds generated a final antibody yield of about 0.60 g.L⁻¹, a 50-60% increase over that observed for unfed culture. Specific productivity, measured over the period of days 0-10 of culture (to retain equivalence to the unfed condition), was enhanced by 25-30% with feed addition (for day 0-10 culture period; specific productivity; Unfed, 10.72 pg.cell⁻¹.d⁻¹, EfficientFeedA, 13.00 pg.cell⁻¹.d⁻¹, EfficientFeedB, 12.36 pg.cell⁻¹.d⁻¹). The increased yield of antibody in the presence of feeds is a consequence of both an increased specific productivity and an enhanced cumulative cell number contributing to overall production. The basic parameters of growth rate and specific productivity are similar to those published for the GS-CHO cell line LB01 and for other antibody-producing CHO cells [24-26]

3.2 Metabolic profiling of medium nutrients under conditions of batch and fed-batch culture

Our model system has shown that CHO CD EfficientFeeds A and B have similar effects of CHO cell growth and influence total cell biomass accumulation whilst presenting an increased yield of recombinant antibody. Having defined the functional consequences of feed for production, our next steps were to analyse extra- and intra-cellular compartments for metabolite changes associated with enhanced growth and productivity to enable interpretation/association of specific metabolic profiles to specific productivity and cell numbers

Metabolites in the extracellular compartment were determined on the GC-MS platform described previously [23]. In the absence of feeds, use of glucose during the exponential phase of cell growth resulted in depletion of extracellular glucose

by day 7 of culture (Figure 1C), a time at which cells had initiated entry into decline phase (Figure 1A). Lactate production was an inverse mirror image of glucose use, and lactate did not increase beyond day 6 of culture (Figure 1D). It is notable that lactate remained relatively constant throughout the subsequent 6-7 days of culture which contrasts with some publications that suggest that the decline phase of batch culture is associated with lactate utilization [27]. The small amount of pyruvate in the medium is used throughout the first 6 days of culture and is depleted at the same stage as glucose (Figure 1E). The addition of CHO CD EfficientFeeds® A and B increased extracellular glucose (by approximately two-fold) Consequently, with approximately the same rate of utilization, medium glucose in fed conditions was not depleted until 10-11 days of culture (Figure 1C). This time course of glucose depletion related to an extended viable cell growth profile but did not appear to be the sole determinant of culture extension as CHO CD EfficientFeed® B, in particular, exhibited a prolonged stationary phase with good viability beyond the complete depletion of medium glucose (Figure 1A). Pyruvate use was similar under unfed and fed conditions (Figure 1E) but dramatic differences were observed for lactate production/use (Figure 1D). In contrast to the profile of lactate observed for the unfed culture, both CHO CD EfficientFeeds® A and B produced decreased lactate production during the later phase of exponential cell growth and a clear switch was noted with lactate re-use from day 7 of culture onwards (Figure 1D). The period of lactate use corresponded to the time in which cells were in a non-growing or stationary phase of culture (Figure 1A). It was unexpected that a general increase in nutrient availability (from feeds) should decrease lactate production and the implications of this, in relation to other

metabolic events and the consequence for cell growth and recombinant protein production, will be developed further in the results described below.

The GC-MS analysis provided a profiling of the relative amounts of multiple components throughout culture in the presence and absence of feeds (16 amino acids are shown in Supplementary Figures S1 and S2). Some amino acids (arginine, cysteine, glutamine, histidine, phenylalanine, tryptophan) were detected in very low abundance, especially in the unfed condition. A general feature from this profiling was that the cessation of growth correlated with depletion of many amino acids to amounts that were undetectable. For example, by day 6 in unfed conditions, asparagine, aspartate, glutamate, tyrosine, and, essential amino acids, isoleucine, leucine, lysine, and valine had attained values close to undetectable (Figures S1 and S2). Depletion of such amino acids, along with glucose, will contribute to the cessation of growth and entry of cells into decline phase. The absolute changes in response to addition of each of CHO CD EfficientFeeds® A and B were not identical (e.g. asparagine, Figure S1) but both feeds increased amino acid amounts by multiple-fold (Figures S1 and S2). Generally, this extended the time before amino acids are depleted and, in most cases, the additional amino acids are used rapidly over the first 6-8 days of culture and then are used at lower rates (during the extended stationary/decline phase observed with feeds) through to 10-13 days of culture, the limit of culture in the presence of feeds. Exceptions to this are asparagine, aspartate, cysteine and tyrosine which were depleted by 6-7 days of culture in the presence of EfficientFeeds® A and B. Profiling of amino acids in unfed and fed conditions has highlighted the relationship between usage of

specific amino acids (for general metabolic use and for protein, lipid and nucleic acid synthesis) and the growth profile. Increased amounts of certain amino acids lead to enhanced rates of use but little change to the time before those amino acids were depleted.

A further group of four amino acids have been considered separately (Figure 2). These include two amino acids – glycine and alanine – that are produced during culture and serine and threonine, both of which can enter the general metabolic pool by the action of specific dehydratases (generating pyruvate and α -ketobutyrate, respectively) [28]. The amount of serine and threonine in the medium was markedly increased in response to addition of either CHO CD EfficientFeeds®. Both pyruvate and α -Ketobutyrate, via oxidative decarboxylation, enters the citric acid cycle at the level of acetyl CoA or succinyl CoA, respectively, and can provide alternative resources to catalyse ATP generation within the mitochondria [29]. The increased availability of serine and threonine and their use as energy feeds for the citric acid cycle throughout the later stages of fed batch cultures presents a likely factor in the prolonged survival of cells under the fed condition (Figure 2C and 2D). Decreased amounts of serine were mirrored by increased amounts of glycine (Figure 2C and 2B). For growing cells, serine offers a major route to support the donation of 1-C units to several biosynthetic pathways, such as the methyl group of thymine, through the formation of N⁵, N¹⁰ methylene tetrahydrofolate catalysed by glycine synthase/glycine cleavage enzyme [10,30]. Increased cell growth and extended survival would be enhanced by elevation and maintenance of the activity of the 1-C

biosynthetic pathway and the stimulation of glycine production in fed culture conditions reflects one aspect of an improved cellular culture process. Finally, the profiles of alanine production are intriguing (Figure 2A). Very little change is seen to medium alanine in unfed conditions but the addition of both feeds generated extensive production of alanine. Although CHO CD EfficientFeed $\square$ A provoked a greater production of alanine than CHO CD EfficientFeed $\square$ B both exhibited similar time courses of production with increased production observed only from day 4 of culture onwards (a time when cells appear to be a similar stage of exponential cell growth whether fed or not, Figure 1A). Alanine production continued until about day 8-9 of culture and then utilization was observed (Figure 2A). In passing it should be noted that feed addition stopped lactate production at day 4 of culture onwards (Figure 1D) and a net utilisation was observed for both lactate and alanine in the later stages of batch culture. Although the switch off of lactate production did not differ between CHO CD EfficientFeed $\square$ A and B, the timing and profiling of changes is suggestive that the “extra” components of feeds (added at the start of culture) have an effect that is “read” at specific stages of culture, perhaps presenting information on phasic changes in cellular phenotype. This will be discussed in more detail in the context of all data from this study.

3.3 Metabolic profiling of intracellular metabolites under conditions of batch and fed-batch culture

Our GC-MS approach identified other metabolites that appeared in medium during culture. These included components of the citric acid cycle (citrate and malate) which were not present in unfed conditions but which showed progressive

appearance from day 5 (citrate) or day 7 (malate) of culture in the presence of CHO CD EfficientFeeds® A and B (Figure 3). Analysis of intracellular samples showed that the intracellular amounts of citrate and malate plus succinate and fumarate decreased from day 5-6 onwards in the unfed cultures, with intracellular amounts close to zero by day 6/7 onwards (Figure 3B, 3D, 3F, and 3H). The decrease in the intracellular content of citric acid cycle intermediates occurred as cells entered decline phase. It was notable that the intracellular content of all four citric acid cycle intermediates was increased in cultures with CHO CD EfficientFeeds® in which cell culture growth and survival was extended. Taken together with the release into the large volume of the extracellular medium, the changes to citrate and malate (no extracellular succinate or fumarate was detected) presents a profound change at the level of the mitochondria and the directional flux of carbon skeletons into alternative fates (TCA cycle oxidation, amino acid transamination, lipid biosynthesis) in response to feeds at a time of high cell viability.

Unexpectedly, a series of “redox sink” metabolites were observed in cell culture medium (Figure 4). Glycerol appeared in culture medium after day 5, and although CHO CD EfficientFeed® B resulted in a more prolonged increase in glycerol, glycerol was produced relatively equally under all conditions, with production slowing as cells entered decline phase (Figure 4C). Intracellular glycerol was also observed with a more continuous formation in the presence of the feeds. Sorbitol and threitol were either not present or at exceedingly low amounts in the absence of feeds but were dramatically increased in the presence of CHO CD

EfficientFeeds[□] A and B (Figure 4A and 4E). Measurement of intracellular extracts showed a similar responsiveness to feeds (Figure 4B and 4F). Interestingly, the appearance of sorbitol in the medium occurred during the extended exponential period of cell growth in the presence of feeds and then declined as cells entered stationary and decline phase.

4 Discussion

4.1 Characterisation of cell growth and antibody production in batch and fed-batch culture

Definition of key metabolic events associated with productive CHO cell phenotype has ramifications beyond medium and feed design and offers the possibility of selection (or cell engineering towards) CHO cell clones that will enable more efficient use of current and (re-designed) future culture media and feeds. Modeling the CHO cell internal metabolic environment opens the potential for optimization of medium platforms to favor growth or production, as appropriate (from a rationalized viewpoint) and to offer insight into the potential for product-specific metabolic settings appropriate to products with differing molecular composition. In our present study, CHO CD EfficientFeeds[□] A or B were added individually at the start of batch culture, to simulate a simple feeding strategy with an enriched medium. In general terms, each feed gave similar advantages to the culture process (Figure 1; with a doubling of cumulative cell number, a 25% increase in specific antibody production and an overall 50-60% increase in antibody yield). These data are similar to those reported by others, with GS-CHO and DHFR-CHO cell platforms [4,6,10]. Whilst increased specific productivity does

support greater antibody accumulation in the presence of feeds, the prolonged growth/stationary phase of culture observed in the presence of feeds is a major facet for the enhanced accumulation of recombinant product (Figure 1). It is notable that (in both the absence and presence of feeds) the cell system continued to produce protein in the stationary phase of culture but that accumulation during this phase of culture was greater when feeds were present.

4.2 Metabolite profiling of glycolytic intermediates and amino acids during batch and fed-batch culture

The usage of a series of amino acids was monitored throughout culture. The data for 16 of these is shown in the Supplementary information associated with this manuscript (Figures S1 and S2). These data suggest some general points of note. For many of the amino acids, supplementation with CHO CD EfficientFeeds® A or B resulted in significantly greater amounts of amino acid. Almost all amino acids derived from feeds were close to depletion by day 12/14 of culture (when cells entered a rapid decline phase). Some were depleted much earlier in the culture period (e.g. asparagine, aspartate, cysteine, tyrosine). In the basal conditions of batch culture we have previously commented upon the importance of asparagine, aspartate and glutamate for the GS-CHO cell platform [6] (and this also explains the low, transient appearance of glutamine in the medium). It is notable, that when feeds had been added, the entry of the culture into stationary phase was associated with the depletion of the above 4 amino acids whilst appreciable amounts of other amino acids remained. We take key interpretations from these data that (a) specific amino acids (that may be platform i.e. GS- or DHFR-specific) may require

continued addition if extended cell growth to high cell density is to be favored (b) specific amino acids may be added in excess in current feed formulations and decreased concentrations may offer cost and process implications/simplifications and (c) separate metabolic profiles can be associated with growth and maintenance. The latter point has implications for design of general medium composition and phase-specific feeding regimes and, whilst the discussion so far has focused on a specific group of amino acids alone, subsequent discussion will return to phase-specific metabolic events associated with maintenance of productive cell cultures over prolonged periods.

There is a widely-accepted view that glucose is a critical nutrient to support the growth and recombinant protein production of CHO cells [9,10,20] and our data would not disagree with this opinion as the timing of glucose depletion in basal or fed conditions was associated with the entry of cells into decline phase (Figure 1C). However, glycolysis *per se*, and the metabolic fate of carbon units derived from glucose, was differentially influenced by the concentration of glucose present. The data presented in Figures 1C and 1D is intriguing. Despite greater amounts of glucose in medium supplemented with CHO CD EfficientFeeds® A or B, lactate production is similar until day 4 of culture. Consequently, despite continued glucose use (which in the unfed culture was associated with continued lactate production), lactate production slows down in cultures supplemented with the increased glucose content provided from CHO CD EfficientFeeds® A and B and is then used. There are a number of publications that have shown that lactate use in recombinant CHO cell cultures may offer an indicator of a productive process but

there is no molecular description of the metabolic events associated with these interpretations [27]. Perhaps the most intriguing aspect of our description of the consequences of addition of CHO CD EfficientFeeds® A and B on lactate production (and later use) is that it takes 4 days of culture before the slowing of production becomes apparent (and a further 3 days before use becomes predominant). These observations point towards a change in the metabolic balance of the CHO cell environment engaged by components (or concentrations of components) within feeds. Detailed elucidation of the metabolic status of the CHO cell at day 4 of culture will define cellular phenotype characteristics associated with good specific productivity and growth rate. Such knowledge will aid development of rationalized media/feeds to match optimal characteristics of desirable metabolic phenotypes that might be attained by CHO cell line variant selection or cell engineering. Whilst the details of components of CHO CD EfficientFeeds® A and B are unknown (and commercially sensitive), and nutrients can have “cell-signaling” actions (e.g. in relation to the mTOR pathway, [31]), there are clear metabolic indicators (discussed below) of events associated with the desirable metabolic phenotype.

Whilst pyruvate may provide an alternative metabolic fate for glucose, our data indicated that this was not the case under addition of CHO CD EfficientFeeds® A and B (Figure 1E). In contrast, alanine was a major product in the presence of CHO CD EfficientFeeds® A and B (more so for Feed A) (Figure 2A). It is again notable that alanine production only became noticeable beyond day 4 of culture and was biphasic with use observed in later stages of culture (from day 9 onwards), a time when lactate use slowed (Figure 1D). These data focus again on a key decision

point (day 4/5, in exponential growth phase) where the internal status of the cell in the presence of CHO CD EfficientFeeds® A and B redirects metabolism away from lactate production (linked to cellular redox status) and towards transamination of pyruvate to alanine (linked to handling of amino acids, aspartate and glutamate, and derivation of keto acids, oxaloacetate and oxoglutarate, that may enable anapleurotic function for the mitochondrial citric acid cycle). We have no explanation for the difference in effectiveness of alanine production of Feed A compared to Feed B but assume this to be a result of differences of components that make up each feed.

Serine and threonine can follow several metabolic fates (dependent on the relative activities of cytosolic and mitochondrial enzymes), including pyruvate (with a potential consequence for alanine production), components of the citric acid cycle (succinyl CoA, acetyl CoA) and glycine (that is associated with folate metabolism and, consequently, many aspects of macromolecular synthesis and directly with glutathione production, essential for many aspects of cell well-being). There was very clear enhancement of glycine production from day 4/5 of culture in the presence of CHO CD EfficientFeeds® A and B (Figure 2B), and this was a reflection of enhanced amounts and usage of both serine and threonine during culture (Figures 2C and 2D, respectively). Whilst serine was depleted by 6 days of culture, threonine was not depleted until day 11, towards the end of the stationary phase of culture. Perhaps in keeping with the differential effects of Feeds A and B on alanine production, cultures with Feed B had less serine and threonine and less glycine production. In keeping with the hypothesis that an increased production of

glycine might be associated with enhanced biosynthesis of glutathione, there was a rapid consumption of cysteine when feeds were added (Figure S2). Whilst glutathione was not directly measured in this work, production of glutathione would be a factor relevant for cell growth and higher productivity and, potentially, a key factor in the ability of feeds to prolong cell survival and recombinant protein productivity. Together these data strongly indicate the importance of series of amino acids (serine, threonine, glycine, cysteine) as precursors for, or indicators of biosynthetic capacity and maintenance of CHO cell functionality during prolonged cultures.

4.3 Metabolite profiling of citric acid cycle intermediates during batch and fed-batch culture

The discussions above have commented upon the manner in which feeds have provided support for cell growth/protein synthesis and well-being (amino acids for synthesis, cofactors for macromolecular synthesis, provision of precursors for the citric acid cycle). In addition, whilst the feeds were added at the initiation of culture, day 4/5 of culture was identified as a time when specific metabolic indicators of feed effectiveness are observed. It is clear from our data that this stage of culture is a time when the feeds enable a healthier intracellular environment, at the level of citric acid cycle function, potentially related to lipid biosynthesis and the handling of cellular redox status. For example, the culture medium of cells without feeds gave very low detection of citrate, fumarate, malate and succinate and intracellular amounts of these intermediates of the citric acid cycle decreased from days 5/6 of culture (Figure 3). In contrast, in the presence

CHO CD EfficientFeeds□ A and B, intracellular amounts of all four intermediates were much greater and were maintained at such values until day 11 of culture (our last sampling time). Citrate and malate appeared in medium with both feeds in readily measurable amounts (Figure 3A and 3G, respectively). Not only are these metabolites direct intermediates of the citric acid cycle but citrate is an intermediate of fatty acid/lipid biosynthesis and both are components of mitochondrial-cytosolic shuttle systems (involved in fatty acid/lipid biosynthesis and redox/amino acid interconversions). Findings presented earlier about lactate use, alanine production, serine and threonine metabolism (and the timing) are linked in time and metabolic interactions to the data on the fates of citrate and citric acid cycle activity. Two recent papers highlight the importance of the cytosol:mitochondrial interface for selection of metabolite usage to support the most efficient balance between usage of metabolic resources whilst enabling appropriate control of redox state in cellular compartments [32,33]. Together these data directly indicate that mitochondrial function (and efficiency) offers a major target to enhance the growth, survival and productivity of recombinant CHO cells, whether by nutrient/feed engineering or via a cell engineering approach.

Increased citric acid cycle function and mitochondrial oxidative capacity has been implicated to arise from the increased provision of intermediates to empower the citric acid cycle. It is clear that major metabolic phenotype changes occur at stages of the batch/fed-batch culture, exemplified at day 4 of culture by the change of lactate metabolism from production to use (a similar change seen in both the presence or absence of feeds). From that moment onwards changes in lactate

production and alanine metabolism were observed. Day 4/5 was also marked by increased production of metabolites that enable recycling of NADH and NADPH (sorbitol, glycerol and threitol), both inside and outside cells (Figure 4). To enable continued glucose degradation via glycolysis, with a limited intracellular pool of NAD⁺, NADH produced in glycolysis requires recycling. As lactate is not produced (or is utilized) alternative redox “sinks” are required. Glycerol (produced from intermediates of glycolysis) offers one such alternative for NADH recycling as do sorbitol and threitol for NADPH recycling to NADP⁺. Additionally, the malate, citrate and glycerol 3-phosphate shuttles couple citric acid cycle function with the movement of NADH redox equivalents into the mitochondria to empower the electron transport chain and generate efficient energy generation. Together these data, and the associated interpretation, indicate a broad change across metabolism of in the CHO cell system with time of culture and response to feed. This manuscript does not address the factors responsible for the regulation but the breadth of changes suggest the potential for environmental regulation of cellular signaling events. Environmental co-ordinators such as mTOR, sensitive to nutrient concentrations and many forms of environmental triggers [31], as well as specific regulation of particular enzymes (e.g. lactate dehydrogenase isoforms [27]) are likely to present a complex, co-ordinated set of regulatory events during phases of culture.

Whilst the production of citrate and glycerol (Figs. 3 and 4) can be related to the redox status of the cell/mitochondria, as noted above, other hypotheses are valid. Both metabolites are indicators of rates of lipid biosynthesis, crucial for cell

growth (plasma membranes) as well as for protein production (endoplasmic reticulum, golgi and secretory vesicles). Increased accumulation may reflect the sustainment of cell growth in the presence of feeds with enhanced synthesis and recycling of the lipid membrane system.

4.4 Concluding remarks

In summary our data enable an integrated vision for the indicators of metabolic processes occurring during batch culture of a recombinant CHO cell line and the relationships of specific feed regimes to enhancement of overall process yield (Figure 5). Incorporation of experimental analyses of this type with MFA data and application to modeling approaches [34, 35, 36] has wide implications for process design, both in a general nature, and by extension, to understanding product-specific process requirements. The expectation of enhancement of recombinant protein expression systems will require robust assessment of cellular status and metabolite-level analyses offer direct indicators for definition of routes of intervention.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

5 References

- [1] Wurm, F.M., Production of recombinant protein therapeutics in cultivated mammalian cells. *Nature Biotechnol.* 2004, 22, 1393-1398.
- [2] Walsh, G.. Biopharmaceutical benchmarks 2010. *Nature Biotechnol.* 2010,28, 917-924.
- [3] Wurm, F.M., CHO Quasispecies—Implications for Manufacturing Processes . *Processes* 2013, 1, 296-311.
- [4] Jostock, T, Knopf, H-P., Mammalian stable expression of biotherapeutics. 2012, *Mthds. Molec Biol.*, 899, 227-238.
- [5] Yu, M., Hu, Z., Pacis, E., Vijayasankaran, N., et al, Understanding the Intracellular Effect of Enhanced Nutrient Feeding: Toward High Titer Antibody Production Process. 2011, *Biotechnol. Bioeng.*, 108, 10789-1088.
- [6] Sellick, C.A., Croxford, A.S., Maqsood, A.R., Stephens, G.M., et al Metabolite profiling of recombinant CHO cells: Designing tailored feeding regimes that enhance recombinant antibody production. *Biotechnol. Bioeng.* 2011, 108, 3025-3031.
- [7] Ma, N., Ellet, J., Okediadi, C., Hermes, P., et al. Single nutrient feed supports both chemically defined NS0 and CHO fed-batch processes: Improved productivity and lactate production. 2009, *Biotechnol. Prog.* 25, 1353-1363.
- [8] Porter, A.J., Dickson, A.J., Racher, A.J. Strategies for selecting recombinant CHO cell lines for cGMP manufacturing: Realising the potential in bioreactors. 2010, *Biotechnol. Prog.*, 26, 1446-1454
- [9] Behjousiar, A., Kontoravdi, C., Polizzi, K.M., In situ monitoring of intracellular glucose and glutamine in CHO cell culture. *PLUS ONE*, 2012, 7, e34512.
- [10] Duarte, T.M., Carinhas, N., Barreiro, L.C., Carrondo, M. J. T. et al , Metabolic Responses of CHO Cells to Limitation of Key Amino Acids, 2014, *Biotechnol. Bioeng.*, 111, 2095-2106.
- [11] Chong, W.,P.,K., Yusufi, F.,N.,K., Lee, D.,Y., Reddy, S.,G., et al, Metabolomics-based identification of apoptosis-inducing metabolites in recombinant fed-batch CHO culture media. *J Biotechnol.* 2011, 151,218-224.
- [12] Dietmair, S., Hodson, M.,P., Quek, L-E., Timmins, N.,K., et al, Metabolite profiling of CHO cells with different growth characteristics. *Biotechnol. Bioeng.* 2012, 109, 1404-1414.
- [13] Carinhas, N., Duarte, T.,M., Barreiro, L.,C., Carrondo, M.,J.,T., et al, Metabolic signatures of GS-CHO cell clones associated with butyrate treatment and culture phase transition. *Biotechnol. Bioeng.* 2013, 110, 3244-3257.
- [14] Sheikholesami, Z., Jolicoeur, M., Henry, O., Probing the metabolism of an inducible mammalian expression system using extracellular isotopomer analysis. *J Biotechnol.* 2013, 164, 469-478.
- [15] Templeton, N., Dean, J., Reddy, P., Young, J.,D., Peak antibody production is associated with increased oxidative metabolism in an industrially-relevant fed-batch CHO cell culture. *Biotechnol. Bioeng.* 2013, 110, 2013-2024.
- [15] Sengupta, S., Rose, S.,T., Morgan, J.,A., Metabolic flux analysis of CHO cell metabolism in the late non-growth phase. *Biotechnol. Bioeng.* 2011, 108, 82-92.

- [16] Chong, W.,P.,K., Thng, S.,H., Hiu, A.,P., Lee, D.-Y., et al. LC-MS-based metabolic characterisation of high monoclonal antibody-producing Chinese Hamster ovary cells. *Biotechnol. Bioeng.* 2012, 109, 3103-3111.
- [17] Dean, J., Reddy, P., Metabolic analysis of antibody producing CHO cells in fed-batch production. *Biotechnol. Bioeng.* 2013, 110, 1735-1747.
- [18] Chong, W.,P.,K., Reddy, S.,G., Yusufi, F.,N.,K., Lee, D.,Y., et al Metabolomics-driven approach for the improvement of Chinese Hamster ovary cell growth: Overexpression of malate dehydrogenase II. *J Biotechnol.* 2010, 147, 116-121.
- [19] Selvarasu, S., Ho, Y.,S., Chong, W.,P.,K., Wong, N.,S.,C., et al, Combined in silico modeling and metabolomics analysis to characterise fed-batch CHO cell culture *Biotechnol. Bioeng.* 2012, 109, 1415-1429.
- [20] Ahn ,W.,S., Antoniewicz, M.,R.. Parallel labeling experiments with [1,2-¹³C] glucose and [U-¹³C] glutamine provide new insights into CHO cell metabolism. *Metab. Eng.* 2013, 15, 34-47.
- [21] Sellick, C.,A., Knight, D., Croxford, A.,S., Maqsood, A.,R., et al, Evaluation of extraction processes for intracellular metabolite profiling of mammalian cells: Matching extraction processes to cell type and metabolite targets. *Metabolomics* 2010, 6, 427-438.
- [22] Sellick, C.,A., Hansen, R., Maqsood, A.,R., Dunn, W.,B., et al, Effective quenching processes for physiologically valid metabolite profiling of suspension cultured mammalian cells. *Analyt. Chem.* 2009, 81,174-183.
- [23] Sellick, C.,A., Hansen, R., Stephens, G.,M., Goodacre, R., et al, Metabolite extraction from suspension-cultured mammalian cells for global metabolite profiling. 2011, *Nat Protoc* 6, 1241-1249.
- [24] Porter, A.J., Racher, A.J., Preziosi, R., Dickson, A.J. Strategies for selecting recombinant CHO cell lines for cGMP manufacturing: Improving the efficiency of cell line generation. 2010, *Biotechnol. Prog.*,26, 1455-1464.
- [25] Luo, J., Vijayasankaran, N., Austen, J., Santury, R., et al, Comparative metabolite analysis to understand lactate metabolism shift in Chinese Hamster ovary cell culture process. *Biotechnol. Bioeng.* 2012, 109,146-156.
- [26] Chen, F., Ye, Z., Zhao, L., Liu, X., et al, Correlation of antibody production rate with glucose and lactate metabolism in Chinese hamster ovary cells . 2012, *Biotechnol. Lett.* 34, 425-432.
- [27] Mulukutla, B. C., Gramer, M., Hu, W-S., On metabolic shift to lactate consumption in fed-batch culture of mammalian cells, 2012 *Metabolic Eng.* 14, 138-149.
- [28] House, J.D., Hall, B.N., Brosnan, J.T., Threonine metabolism in isolated rat hepatocytes. 2001, *Amer. J. Physiol.* 281, E1300-1307.
- [29] Edgar, A.J., Cloning and tissue distribution of mammalian L-threonine 3-dehydrogenases. 2002. *BMC Biochem.* 3, 19.
- [30] Narkewicz, M.R., Sauls, S.D., Tjoa, S.S., Teng, C., et al. Evidence for intracellular partitioning of serine and glycne metabolism in Chinese hamster ovary cells. 1996, *Biochem. J.* 313, 991-998.
- [31] Proud, C.G., Amino acids and mTOR signaling. 2007, *Biochem. Soc. Trans.*, 35, 1187-1190.

- [32] Zagari, F., Jordan, M., Stettler, M., Broly, H., et al, Lactate metabolism shift in CHO cell culture: the role of mitochondrial oxidative activity. 2013, *New Biotech.* 30, 238-245.
- [33] Wahrheit, J., Niklas, J., Heinzle, E., Metabolic control at the cytosol-mitochondria interface in different growth phases of CHO cells, 2014, *Metab. Eng.*, 23, 9-21.
- [34] Nolan, R.P., Lee, K., Dynamic model of CHO cell metabolism . 2011, *Metab. Eng.*, 13, 108-124
- [35] Martinez, V.,S., Dietmair, S., Quek, L-E., Hodson, M.,P., et al, Flux balance analysis of CHO cells before and after a metabolic switch from lactate production to consumption. *Biotechnol. Bioeng.* 2012, 110, 660-666.
- [36] Chen, N., Bennett, M.H., Kontoravdi, C. Analysis of Chinese hamster ovary cell metabolism through a combined computational and experimental approach. 2014, *Cytotechnology*, 66, 945-966.

Figure legends

Figure 1. Influence of CHO CD EfficientFeed A (Feed A) or CHO CD EfficientFeed B (Feed B) on growth (panel A), antibody production (panel B), medium glucose (panel C), medium lactate (panel D) and medium pyruvate (panel E) of GS-CHO LB01 cells during batch culture. Cell culture and analytical processes for cell number, antibody concentration and metabolite quantitation are described in Materials and Methods. Basal medium was CD-OptiCHO (Unfed, ● circle) and when present feeds (Feed A, ■ square; Feed B, ▲ triangle) were added once at the start of the culture. Glucose, lactate and pyruvate in medium were determined with the GC-MS analytical platform and values are arbitrary units normalized to an internal standard. All data represents the average of six replicates, with standard deviation error bars.

Figure 2. Influence of CHO CD EfficientFeed A (Feed A) or CHO CD EfficientFeed B (Feed B) on metabolism of medium alanine (panel A), medium glycine (panel B), medium serine (panel C) and medium threonine (panel D) by GS-CHO LB01 cells during batch culture. Cell culture and analytical processes for metabolite quantitation are described in Materials and Methods. Basal medium was CD-OptiCHO (Unfed, ● circle) and when present feeds (Feed A, ■ square; Feed B, ▲ triangle) were added once at the start of the culture. Metabolites in medium were determined with the GC-MS analytical platform and values are arbitrary units normalized to an internal standard. All data represents the average of six replicates, with standard deviation error bars.

Figure 3. Influence of CHO CD EfficientFeed A (Feed A) or CHO CD EfficientFeed B (Feed B) on amounts of components of the citric acid cycle in extracellular and intracellular compartments of GS-CHO LB01 cells during batch culture. Cell culture and analytical processes for extracellular (panels A, C, E and G) and intracellular (panels B, D, F and H) metabolite quantitation are described in Materials and Methods. Basal medium was CD-OptiCHO (Unfed, ● circle) and when present feeds (Feed A, ■ square; Feed B, ▲ triangle) were added once at the start of the culture.

Metabolites (citrate, panels A and B; succinate, panels C and D; fumarate, panels E and F; malate, panels G and H) were determined with the GC-MS analytical platform and values are arbitrary units normalized to an internal standard. All data represents the average of six replicates, with standard deviation error bars.

Figure 4. Influence of CHO CD EfficientFeed A (Feed A) or CHO CD EfficientFeed B (Feed B) on amounts of sorbitol, glycerol, threitol and inositol in extracellular and intracellular compartments of GS-CHO LB01 cells during batch culture. Cell culture and analytical processes for extracellular (panels A, C, E and G) and intracellular (panels B, D, F and H) metabolite quantitation are described in Materials and Methods. Basal medium was CD-OptiCHO (Unfed, ● circle) and when present feeds (Feed A, ■ square; Feed B, ▲ triangle) were added once at the start

of the culture. Metabolites (sorbitol, panels A and B; glycerol, panels C and D; threitol, panels E and F; inositol, panels G and H) were determined with the GC-MS analytical platform and values are arbitrary units normalized to an internal standard. All data represents the average of six replicates, with standard deviation error bars.

Figure 5. Profiling of key metabolic indicators of recombinant CHO cell processes in batch and fed-batch cultures. This Figure provides an integrated summary points discussed in the text. Arrows indicate the direction of reactions/transport with double arrowheads indicating the reaction/transport is reversible. Standard amino acid abbreviations are used.