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**CHO-K1 host cells adapted to growth in glutamine free medium
by FACS-assisted evolution**

Juan A. Hernández Bort¹, Beate Stern² and Nicole Borth¹

¹Department of Biotechnology, University of Natural Resources and Applied Life Sciences,
Vienna, Austria

²UniTargetingResearch AS, Bergen, Norway

Corresponding Author: Nicole Borth
University of Natural Resources and Applied Life Sciences
Department of Biotechnology
Muthgasse 18
1190 Vienna
Austria
Tel. +43 1 47654 6232
Fax: +43 1 369 7615
Email: nicole.borth@boku.ac.at

ABSTRACT

During the process of recombinant cell line optimisation for production of biopharmaceuticals, multiple cellular properties like robustness against stress, the attainment of high cell concentrations and maintenance of high viability must be considered in order to maximize protein yield. To improve growth and viability, glutamine is supplemented as an alternative energy source for rapidly dividing cells that oxidize glucose inefficiently. However, the resulting by-product ammonia is toxic at high concentrations and has a negative impact on protein glycosylation, a major quality determining parameter of biopharmaceuticals. In this work, the CHO-K1 cell line was adapted to a chemically defined medium and suspension growth within 3 weeks. Subsequently, the glutamine concentration was stepwise reduced from 8mM to 4mM and 2mM. After each reduction, both the final cell concentration in batch and the viability decreased. In order to force a rapid evolution of cells to achieve high final cell concentrations, cells were seeded at high densities (10^7 cells/ml) and surviving cells were sorted by FACS or MACS when viability declined to 10% (typically after 24h). Sorted cells were grown in batch until viability declined to 10% and viable cells recovered again. The final sorted population was able to reach comparable or even better viable cell concentrations and showed a significantly improved viability compared to their ancestors. The 2mM glutamine-adapted cell line was directly transferred into glutamine-free medium and was able to grow at comparable rates without requiring further adaptation. Cells compensated the lack of glutamine by increasing their consumption of glutamate and aspartate.

KEYWORDS

glutamine; ammonia; CHO-K1 cells; cell sorting; assisted evolution; glutamine-free medium

INTRODUCTION

Mammalian cells, and especially Chinese hamster ovary (CHO) cells, are widely used in biomedical research and the pharmaceutical industry for production of recombinant proteins. Thus, the improvement of cellular properties to increase productivity and product quality is of major interest.

During the last decades many studies were carried out in order to improve growth media. Glucose and glutamine concentrations are usually overdosed in mammalian cell culture media to achieve high growth rates and biomass concentration. However, such high concentrations lead to higher consumption rates of these components [1-3]. On the one hand, entry of glucose into the glycolytic pathway leads to pyruvate formation, which can either be shuttled into the tricarboxylic acid (TCA) cycle or converted into lactate. Due to the high flux of glucose to pyruvate and the inefficient coupling between glycolysis and TCA cycle, lactate accumulates during cultures, which has a negative impact in cells [3-5]. On the other hand, glutamine is the amino acid consumed with the highest rate and its importance for an optimal growth in cell culture has been known since the 50s [6]. Glutamine is an alternative energy source to glucose for rapidly dividing cells, as it is mostly metabolized via a truncated form of the TCA cycle [7]. Feeding of excess glutamine induces a high ammonium production in cell cultures [1, 8], which reduces viable cell density and productivity [4, 9-11] and alters glycoforms of proteins [12-15]. Moreover, it was also described that ammonia concentrations of 10mM will prevent growth of fibroblasts [16]. Although ammonia is derived mainly from deamidation of glutamine [6], the catabolism of the other amino acids present in the cultivation medium is also responsible for its overproduction [17]. When ammonium diffuses across the cell membrane, intracellular pH (pH_i) tends to decrease and electrochemical gradients are perturbed [18, 19]. To overcome this problem, glutamine has been substituted by other less ammoniagenic carbon substrates like glutamate or asparagine

[20, 21]. In addition, sophisticated methods, including microporous hollow fiber modules or media filtration with cation exchangers, have been developed for waste product reduction in culture supernatants, in order to maximize the final product concentration and yield [22-24]. Substitution of glucose and glutamine by other energy substrates successfully reduced lactate and ammonia, respectively, but resulted in considerably decreased viable cell density [25]. However, it was also described that control of glutamine at low levels can improve cell densities [26].

For several years now, growth media for CHO cells have been optimised to a degree that allows growth in protein-free media [27-31]. In addition, cells have even been subjected to “Bioreactor evolution” [32] to improve their process relevant properties. All in all, increases in volumetric productivity during the last 10 years were mainly achieved by increases in the integral viable cell density (IVCD), by optimising process design and feeding strategies [33-35]. Additional improvements were obtained by prolonging cell survival at the end of the culture by decreasing cell sensitivity to apoptotic stimuli. This strategy was tried by expression of anti-apoptotic genes in monoclonal antibody producing CHO cells [36-39].

In an attempt to obtain an optimised host cell line to be used for recombinant biologic production, we explored the possibility to achieve comparable growth and viability in a cell line independent of glutamine in the culture medium. In the present report we present a new approach to easily and rapidly evolve cells towards improved growth properties using cell sorting assisted evolution. Cells surviving a progressive stress treatment were sorted out of the parent population based on their cellular state and viability and typically showed enhanced robustness, a prolonged life span and higher viable cell density with concomitant improvement in IVCD.

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MATERIALS AND METHODS

Cell line and media

The original CHO-K1 adherent cell line obtained from the American Type Culture Collection (ATCC) was adapted within three weeks to growth in suspension in CD CHO, a chemically defined, serum-free medium (Gibco, Invitrogen, Carlsbad, CA, USA) and supplemented with 8mM L-glutamine. Studies were carried out in spinner flasks with a working volume of 100ml at 37°C in a humidified atmosphere containing 7% carbon dioxide and with constant stirring at 50rpm.

Progressive reduction of glutamine and Assisted Evolution

Cells were seeded in a concentration of 10^7 cells/ml incubated at 37°C and surviving cells were sorted out with either Fluorescence Activated Cell Sorting (FACS) or Magnetic Activated Cell Separation (MACS) when viability declined to 10% after 20-24 hours. Surviving cells were expanded and seeded into a spinner flask with a starting concentration of 1×10^5 cells/ml. The batch was monitored until viability dropped below 10%. Surviving cells were sorted out again and transferred into medium with reduced glutamine concentration. This procedure (Fig. 1) was repeated reducing glutamine supplement into culture medium after each completed cycle from 8mM to 4mM, and from 4mM to 2mM. Finally, cells adapted to 2mM glutamine were transferred into culture medium without glutamine supplementation.

Viability and cell concentration

Cell viability was estimated with a Neubauer hemocytometer using the trypan blue exclusion method. Total cell number was determined by counting cell nuclei with a Multisizer™ 3

Coulter Counter (Beckman Coulter, Fullerton, CA, USA) after lysis of the cell membrane by incubation of samples for two hours in 0.1M citric acid containing 2% Triton X-100.

Cell sorting

Viable cells were sorted out from cell culture bulk using either a FACS Vantage™ (Becton Dickinson, Franklin Lakes, NJ, USA), equipped with a Sort Enhancement Module, or a Dead Cell Removal kit in combination with a MACS column (Miltenyi Ltd, Germany). MACS protocol was performed according to supplier instructions. For FACS, a combination of a SSC-H / FSC-H gate and a gate for cells that exclude DAPI (4,6-diamidino-2-phenylindole) was used. DAPI is taken up and stains DNA only of damaged or dead cells. The intact sorted cells were deposited into a 6-well plate containing 3ml growth medium pre-incubated at 37°C. Both cell sorting systems were tested and found to give similar results, with the MACS protocol faster and easier to handle. The FACS protocol is more time consuming, but due to its multiparameter character allows a finer discrimination between healthy cells and cells, that although still with an integral membrane, are already compromised.

Analysis of glucose, lactate and ammonia.

Concentration of glucose and lactate in the cell culture supernatant were determined using a YSI 7100 MBS Bioanalytical System (YSI Life Sciences, USA). Ammonia was measured by photometer with an Ammonia Assay Kit (Megazyme, Ireland) according to supplier's instructions.

Amino acid analysis

A HPLC Agilent 1200 Serie System equipped with a fluorescence detector (Ex:340nm; Em:450nm) was used for amino acids determination of the cell culture supernatant harvested

every 4 days, including day 0. A volume of 30 μ l sample mixed with 20 μ l ortho-phthalaldehyde (Pierce), was injected on a reversed phase Hypersil ODS column (3 μ m particle size, 4.0x100mm) thermostated at 25°C, with the following linear gradient of sodium acetate 50mM, pH 5.70 (solvent A) and acetonitrile (solvent B): the concentration of 6% B was kept constant for 2 min., then the concentration of B was increased linearly from 2 to 12 min. to 9% B, from 12 to 18 min. to 19% B, from 18 to 26 min. to 22% B, from 26 to 38 min to 42% B and from 38 to 40 min to 100% . Column equilibrium to original conditions was from 40 to 50 min to 6% B. A flow rate of 1.5ml/min. was used throughout. Peaks were identified by retention time and concentrations determined relative to 3-(2-thienyl)-DL-alanine (Fluka), an internal standard added to each sample. Calibration range was from 1mg/l to 25mg/l.

Plasmid and transient transfection

For transient transfection, 10⁷ cells were co-transfected with 5pmol each of two plasmids bearing a human IgG1 heavy chain and lambda light chain under the control of a cmv-promoter. For the calculation of molar plasmid concentrations, the molecular weight average of a nucleotide pair was assumed to be 660 pg/pmol. Only cells with viability greater than 95 % were used for transfection. According to Amaxa AG protocol, cell pellet was resuspended in 100 μ l pre-mixed Solution kit V + Supplement (both included in Amaxa kit V), mixed with plasmids, and immediately nucleofected with the Amaxa Nucleofector (programm H-14). Directly after the electroporation procedure, cells were transferred into a culture flask with growth medium pre-warmed to 37°C at a concentration of 2x10⁵ cells/ml. Samples were harvested to determine viability and starting cell number. Final cell density and viability were determined 48 hours after transfection. The protocol was previously evaluated to yield transfection efficiencies better than 90 and to have a standard deviation of +/- 15% [40]

IgG heavy chain determination by ELISA

Microtiter Nunc MaxiSorp were coated with goat-anti-human-IgG-gamma-chain-specific (#I3382; Sigma) diluted 1:1000 in 0.1M sodium bicarbonate buffer (pH=9.6) at 4°C overnight. The plates were washed and then incubated with serial dilutions of a self-purified IgG standard and culture supernatant samples for 1 hour in a dilution buffer containing 1% BSA at room temperature. After a wash step, the bound IgG was incubated with horse radish peroxidase (HRP) conjugated with a goat-anti-human-IgG (#62-8420, Zymed) diluted 1:1000 in dilution buffer for 1 hour. For the detection of the antigen-antibody reaction, 200µl of a 0.4mg/ml o-phenyldiamine dihydrochlorid (OPD) solution was added as substrate after a wash step, and incubated for 5 minutes at room temperature. The OPD reaction was stopped with 50µl of 2M H₂SO₄ and read at 492nm. Calculation was carried out using a 4 parameter calibration curve with a detection range from 0 to 200ng/ml IgG. Deviation of standard, control and sample replicates was less than 15%.

RESULTS

Extension of culture life span

The rationale for this work arose from the concept that cells submitted to an iterative but not drastic change of milieu conditions should be able to evolve towards a more efficient metabolism and better adaption to the new conditions. When glutamine supplement was reduced in CHO-K1 culture medium without adaptation, both growth rate and viable cell density decreased by approximately 20% (data not shown). This reduction became even worse during further routine passage in medium with the reduced glutamine content for a period of at least 6 weeks. In order to quickly restore cellular growth properties, cells were

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3 subjected to an iterative process of selection for the best adapted cells in reduced-glutamine
4 culture medium.
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8 To achieve this, CHO-K1 cells were seeded at high density (10^7 cells/ml) and incubated at
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10 37°C until viability declined to 10%. This typically occurred after 20-24h. Surviving cells
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12 were sorted out with FACS or MACS and expanded in a T flask. This was necessary to
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14 remove dead cells and debris, which at high concentrations is toxic and would have killed the
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16 few remaining viable cells. In the second part of this approach, a batch with the expanded
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18 cells was started by seeding cells at 1×10^5 cells/ml in a spinner flask. The batch was
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20 monitored until viability dropped below 10%. Surviving cells were sorted out. The process
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22 was repeated, while reducing glutamine supplement after each completed cycle from 8mM to
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24 4mM, and from 4mM to 2mM. Finally, cells adapted to 2mM glutamine were transferred into
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26 culture medium without glutamine supplementation and showed the same growth profile as
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28 cells supplemented with 2mM glutamine. Thus for the 0mM glutamine adapted cell line no
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30 additional cycle in assisted evolution was required. The different adapted cell lines were
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32 maintained in the medium with the respective glutamine concentration in routine culture (on
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34 total now for over one year, without loss of phenotypic properties, data not shown).
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41 In order to compare changes in cellular properties, batch cultures with these newly
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43 established CHO-K1 cell lines were started with the respective L-glutamine concentrations
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45 (8mM, 4mM, 2mM and 0mM) in spinner flasks with a working volume of 100ml and an
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47 initial concentration of 1×10^5 cells/ml, incubated at 37°C and 7% CO₂ with constant stirring
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49 at 50rpm. Results presented are the mean of two independent replicate batch cultures.
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53 The 2mM and 0mM adapted cell line was able to increase the life span by approximately
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55 30% relative to the parental cells and to maintain a viability of greater than 95% for 10 days
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57 (Fig. 2). Moreover, the integral of viable cells was increased by 22% for the 2mM culture and
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59 by 13% for the 0mM culture (Fig. 3).
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Cellular Metabolism

To evaluate the changes in metabolism that occurred upon assisted evolution, the adapted cell lines were cultivated in batch cultures in duplicate and the nutrient consumption of amino acids and glucose, as well as production of lactate (Fig. 4) and ammonium was analysed. The cells adapted to 2mM and 0mM glutamine, although lacking an important energy source, did not increase their glucose uptake rate nor did they shift towards more efficient utilization of glucose, as the ratio of lactate produced per glucose consumed remained the same. Instead, these cells compensated for carbon and nitrogen source by increasing the consumption of glutamate, aspartate and to a lesser degree asparagine (Fig. 5). Aspartate is produced in the cultures grown at 8 and 4mM, while it is consumed in those at 2 and 0mM. Uptake of other amino acids was slightly lower, while the production rate of alanine and to a lesser degree glycine was lower in these cultures than in the cultures grown at 8 and 4mM glutamine. Except for glutamine, no amino acid was completely depleted at the end of batch cultures. The low alanine secretion and ammonium production (Fig. 6) in culture supernatant by cells adapted to reduced-glutamine media during growth phase agrees with the conclusions of some authors, who reported that excess of ammonium production may be the cause of high alanine excretion after activation of transamination pathways [5, 9, 41].

Final ammonia concentration in CHO-K1 2mM grown at 0mM glutamine was comparable to CHO-K1 8mM and CHO-K1 4mM (Fig. 6), in spite of the absence of glutamine in growth medium. On the other hand, a reduction in ammonia production was observed during the exponential phase of batches.

Growth rate and integral viable cell density

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Comparison between adapted and non-adapted cell lines revealed differences in specific growth rates. As highlighted in Fig. 2, CHO-K1 2mM and CHO-K1 0mM cell lines had a delayed start of exponential phase compared to cells adapted to growth in 8mM and 4mM glutamine. Moreover, 2mM glutamine-adapted cells had a prolonged growth phase with respect to their parents and the viable cell density was increased. This improvement was reflected in the cumulative viable cell density, particularly in the CHO-K1 2mM cell line (Fig. 3).

Effects on transient expression of IgG

Previous studies have shown that glutamine reduction in cell culture media reduced protein production [25]. CHO-K1 8mM, 4mM 2mM and 0mM cells were transfected with a total of 10pmol plasmid encoding IgG heavy chain and light chain genes to verify if any immediate effect on the specific productivity could be observed. The IgG product secreted in culture supernatant was quantified by ELISA at day 2 after transfection. Specific productivity reached 48h after transfection was comparable in all cases (Fig. 7), considering the standard deviation of the method, which was +/- 15%.

DISCUSSION

Survival of the best adapted organism to a new environment is generally known as natural selection. In an attempt to transfer the Darwinian Theory to an optimisation procedure for CHO-K1 cells, a simple strategy to improve properties of CHO-K1 was developed. Cells were submitted to an iterative and progressive alteration of milieu conditions by bulk selection of best adapted cells.

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Previous studies have shown that after decreasing the glutamine concentration in growth media to 0mM, cellular metabolism shifted toward glycolysis leading to a higher glucose consumption rate [42]. Because glutamine is an important source for cellular glutamate, its reduction could act as a rate-limiting step in the synthesis of glutathione (GSH), a tripeptide consisting of glutamate, glycine, and cysteine, that serves to protect cells from oxidative stress [43, 44]. However, the specific glucose uptake and lactate production rates in the cell lines adapted to lower glutamine concentrations were not significantly changed, nor was the ratio of lactate produced per glucose consumed. The only difference was that in the culture grown without glutamine the cells were able to consume glucose down to a concentration of less than 200mg/l (Fig. 4).

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A comparison of ammonia production (Fig. 6) revealed an initially dose dependent reduction in ammonia production with decreasing glutamine concentrations during exponential growth. On day 8 and 12 the ammonia concentrations were however comparable due to increased uptake of other amino acids in the cells grown at reduced glutamine concentrations. This result contradicts the intention of reducing glutamine to improve the glycosylation properties of produced proteins at later stages of the production process. In previous work on reducing or replacing glutamine [20, 23] the actual ammonia concentration was never tested. Nevertheless, by weening the cells of glutamine, we have achieved an increase in the integral of viable cell days (IVCD) of 20%, this in spite of the prolonged lag during in early batch culture. The IVCD is the main determining factor for total product yield and thus economic process output. The next step in determining the usefulness of this new host cell line, will be to repeat the process with a producer cell line and verify effects on total high level productivity as well as on product quality.

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CONCLUSION

We presented a new approach to easily and rapidly evolve cells towards improved growth properties using a combination of Assisted Evolution Approach and cell sorting. Cells surviving a progressive stress treatment were sorted out of the improved parent population and typically showed improved robustness, a prolonged life span and enhanced viable cell density. The whole procedure of adapting cells from 8mM glutamine containing medium to 0mM glutamine took less than 10 weeks.

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Figure 1

Assisted Evolution Approach. Glutamine concentration in media was reduced only after each completed cycle.

Figure 2

Profiles of growth (A) and viability (B) of CHO-K1 adapted to different glutamine concentrations with an initial cell density of 1×10^5 cells/ml.

Figure 3

Cumulative integrated viable cell density during the 12-day-batches. CHO-K1 2-2 and 2-0 cell lines increased IVCD after Assisted Evolution.

Figure 4

(A) CHO-K1 2-0 metabolised glucose slower than compared cells lines in spite of lack of glutamine in growth medium. (B) Lactate production correlated with glucose consumption.

Figure 5

Different consumption and production profiles of selected amino acids during performed batch cultures. (A) Glutamine, (B) Glutamate, (C) Aspartate, (D) Asparagine, (E) Alanine, and (F) Phenylalanine.

Figure 6

Final ammonia concentration at the end of batch cultures was similar in all cell lines.

Figure 7

Specific production rate (q_p) of different CHO-K1 cells adapted to 8mM, 4mM and 2 mM glutamine cultivated in different glutamine concentrations: 8mM, 4mM, 2mM and 0mM. Cell concentration and IgG titers in the supernatant were determined at time 0h and 48 h after transfection with 10pmol plasmid DNA.

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257x318mm (300 x 300 DPI)

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