DirectedCHO: A new miniaturized directed evolution process for phenotype stability trial test of CHO cells before bioreactor scale-up

Deepak B. Thimiri Govinda Raj *, N Musasira, MM Takundwa

Synthetic Nanobiotechnology and Biomachines Group, Synthetic Biology and Precision Medicine Centre, Next Generation Health and Chemical Cluster, CSIR Pretoria, South Africa

ABSTRACT

Most of the biopharmaceuticals that are currently on the market are expressed using the Chinese Hamster Ovary (CHO) cell lines. However, the production yield of these biopharmaceuticals is affected due to CHO cellular heterogeneity and challenges in adaptability during the bioreactor scale-up stage. In this communication, we report the protocol for the miniaturized directed evolution process for CHO cells. The results of the directed evolution process would guide adapting the CHO cell line before bioreactor scale-up. With our approach, we have established the protocol that can be used to streamline superior CHO cell lines for biopharmaceutical production which would be the first of its kind in Africa. Our directed evolution protocol includes a method for a low-cost multiplex directed evolution process that can be used on CHO cells using 20 stressors in 8 concentrations and provides stable trial results for the scale-up process. Using our process, we can provide a simple consumable kit that manufacturers can use for the CHO cell phenotype stability test before the scale-up process. With our approach, we would further develop a platform that can streamline superior CHO cell lines for biopharmaceutical production. This approach would be the first of its kind in South Africa/ Africa.

1. Introduction

It has been reported that the biopharmaceutical industry has an annual growth of 18 % (the highest among biotechnology industries) and 350 biopharmaceuticals are currently in clinical trials or market [1]. Various cell factories such as E.coli, S. cerevisiae, insect cells, and mammalian cell lines like Chinese Hamster Ovary (CHO) cell lines have been used for the commercial manufacture of biological drugs [1]. At present, over 70 % of those on the market are manufactured using Chinese Hamster Ovary (CHO) cells; However, the current demand for biopharmaceutical products will change this trend.

The increase in the need for the production of complex biomolecules further deteriorates the CHO-based biomanufacturing economics as we prepare for more futuristic applications [2] [multiple drugs being manufactured simultaneously, point-of-care drug manufacturing, drugs being medicine requirements for space migrants, etc.]. A key characteristic contributing to the limitations of CHO cell lines is their heterogeneity. The main focus for biopharmaceutical manufacturing is the robust strategy for cell line development through which we choose the cell line with the best bioproduction properties for that specific drug of interest. Obtaining a parental cell line [starting material for which the DNA of the drug to be manufactured is inserted] that inherently exhibits superior ‘generic’ attributes would therefore be highly desirable e.g. folding capabilities, superior glycosylation capacity [3–6]. An alternative approach is direct evolution processes to the creation of ‘generically’ improved mammalian cell hosts. Several research groups have attempted to improve production yield with limited success [7–9].

Valitacell has an award-winning platform – “Chemstress™ fingerprinting” that uses small molecule chemical stressors to simulate the stresses that clones will experience in a bioreactor. Clones that perform well in bioreactors have the desired bioproduction characteristics including compensatory mechanisms to overcome bioreactor stressors encountered. Over many years Valitacell has developed a unique understanding of the compensatory metabolic processes of clones in bioreactors and has designed a high-throughput analytical technology that ‘simulates’ these bioreactor stresses using small molecules. They have furthermore demonstrated that by measuring various cell outputs e.g. growth, productivity, etc., in response to these stressors this information, the ‘Chemstress™ fingerprint’, be used from a regulatory perspective for clone authentication and it can also be used to predict the performance [productivity and stability] of that clone in a scaled-up bioreactor [10,11]. Through Chemstress TM, we have learned a lot about what an ideal CHO ‘Phenome’ looks like and how it should behave.

However, the Chemstress technology can only provide the phenotype fingerprint profile on the impact of bioreactor stressors on the CHO cells for a period of three days of culturing. The Chemstress technology cannot provide the real-time phenotype fingerprint profile of CHO cells...
in the presence of bioreactor stressors for more than three days. There is currently an unmet need to develop a multiplex methodology that would perform the directed evolution process of CHO cells in the presence of bioreactor stressors. Furthermore, there is a critical need to develop several strategies for generating superior CHO cell lines with improved biopharmaceutical production yield along with reduced time and cost of manufacturing. One of the strategies includes the establishment of miniaturized culturing of suspension CHO cells using deep-well plates. Here we report our methodology where we developed a simple consumable kit on which biomanufacturers can carry out directed evolution on existing CHO hosts that would enable enhanced yield as well as reduced production cost. Our methodology includes rewiring the Chemstress into EVO plates and performing directed evolution in a deep-well culture setting. Our manuscript also details the technical approach for generating superior CHO cell lines with improved biopharmaceutical production yield along with reduced time and cost of manufacturing.

Table 1
Chemstress ™, function and its application.

<table>
<thead>
<tr>
<th>Chemstress ™ drugs</th>
<th>Function</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Deoxy d-glucose</td>
<td>Metabolism modulators</td>
<td>2-deoxy-d-glucose (2DG) is known as a synthetic inhibitor of glucose</td>
</tr>
<tr>
<td>Ammonium Chloride</td>
<td>Toxic</td>
<td>Effects of ammonium and lactate on growth and metabolism of a recombinant Chinese hamster ovary cell culture.</td>
</tr>
<tr>
<td>3-Amino-1,2,4-triazole (AMT)</td>
<td>Metabolism modulators</td>
<td>Effects of inhibition of catalase activity on metabolism</td>
</tr>
<tr>
<td>5-Azacytidine</td>
<td>Cell Cycle modulators</td>
<td>5-Azacytidine is a well-studied methyltransferase inhibitor, known to activate gene expression</td>
</tr>
<tr>
<td>L-buthionine sulfoximine</td>
<td>Oxidative stress</td>
<td>to study oxidative stress in CHO cells producing recombinant protein, induce glutathione limitation using buthionine sulfoximine (BSO) treatment</td>
</tr>
<tr>
<td>2-Amino-2-norbornane carboxylic acid</td>
<td>Nutrient starvation</td>
<td>The leucine analog, 2-amino-2-norbornanecarboxylic acid (BCH) is a highly specific transportable inhibitor for LAT1–4. System L Transport inhibition.</td>
</tr>
<tr>
<td>Brefeldin A</td>
<td>Nutrient starvation</td>
<td>ER Stress. BFA is a fungal fatty acid metabolite first used experimentally as an inhibitor of viral replication, and later shown to inhibit protein secretion via specific pathways of intracellular vesicular trafficking.</td>
</tr>
<tr>
<td>Cadmium acetate * 2 H2O</td>
<td>Cell Cycle Modulators, Apoptosis mediators</td>
<td>intracellular catalase is important to prevent the formation of oxidative DNA damage as well as deletions and GC→AT transitions upon cadmium exposure.</td>
</tr>
<tr>
<td>Citric acid</td>
<td>pH &amp; osmolarity stress</td>
<td>Energy pathway metabolites Citric acid</td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td>Oxidative stress</td>
<td>A Chemical hypoxia-mimicking agent,</td>
</tr>
<tr>
<td>D-Phenyllalnine (D-Phe)</td>
<td>Nutrient starvation</td>
<td>Poly-pathway model, a novel approach to simulate multiple metabolic states by reaction network-based model-Application to amino acid depletion in Cho cell culture</td>
</tr>
<tr>
<td>α-Methylamino-isobutyric acid (MeAIB)</td>
<td>Nutrient starvation</td>
<td><a href="https://pubmed.ncbi.nlm.nih.gov/7,880,975/">https://pubmed.ncbi.nlm.nih.gov/7,880,975/</a></td>
</tr>
<tr>
<td>Menadione sodium bisulfite</td>
<td>Oxidative stress</td>
<td>Menadione sodium bisulfate (MSB) Site-2 protease responds to oxidative stress and regulates oxidative injury in mammalian cells.</td>
</tr>
<tr>
<td>Sodium butyrate (NaBu)</td>
<td>Cell Cycle modulators</td>
<td>Sodium butyrate (NaBu) is not only known to inhibit proliferation but also to increase the specific productivity of protein production</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>pH and osmolarity stress</td>
<td>To understand the transcriptional responses to increased osmolarity and relate changes in gene expression to increased productivity and repressed growth, proprietary CHO microarrays were used to monitor the transcription profile changes in response to osmotic stress</td>
</tr>
<tr>
<td>Sodium l-lactate (NaLac)</td>
<td>Cell Cycle modulators</td>
<td>Sodium lactate is the sodium salt of L(+)-lactic acid, obtained by neutralization of the acid of natural origin with a high-purity sodium source.</td>
</tr>
<tr>
<td>Sodium orthovanadate (NaOtvh)</td>
<td>Metabolism modulators</td>
<td>Sodium Orthovanadate (Vanadate) is a general competitive inhibitor for protein phosphorytose phosphatases.</td>
</tr>
<tr>
<td>Sodium oxamate (NaOxam)</td>
<td>Metabolism modulators</td>
<td>Oxamate, but Not Selective Targeting of LDH-A, Inhibits Medulloblastoma Cell Glycolysis, Growth and Motility</td>
</tr>
<tr>
<td>Rapamycin (Rapa)</td>
<td>Nutrient starvation</td>
<td>Valproic acid can serve as an alternative to sodium butyrate for enhancing protein expression in mammalian cell cultures</td>
</tr>
<tr>
<td>Valproic acid sodium salt (Val)</td>
<td>Metabolism modulators</td>
<td>VPA is both FDA-approved and 5-fold less expensive than sodium butyrate (NaBu) and is a cost-effective alternative enhancer of recombinant protein production</td>
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Fig. 1. Chemstress fingerprint profile of CHO cells.
advancement to convert the ChemStress technology which can be performed only in adherent cells to also be used for suspension cells. This is achieved using deep-well culturing and the implementation of Evo plates for directed evolution.

2. Materials and methods

2.1. Culturing of suspension CHO cell culture

We received suspension CHO-K1 cells from Valitacell that were frozen using Synth-a-Freeze™ Cryopreservation Medium with a cell density of $1 \times 10^7$ cells/ml. Suspension CHO-K1 cells are revived using CD-CHO medium supplemented with antibiotics (Penicillin and Streptomycin) and cultured in a 125 ml Shaker flask without FBS. Cells are cultured at $37^\circ$C on CO$_2$ resistant orbital shaker at 135 rpm in a CO$_2$ incubator setup.

Subculturing of suspension CHO-K1 cells can be seeded at $2 \times 10^5$–$3 \times 10^5$ viable cells/mL into sterile shake flasks containing pre-reduced CD CHO Medium (30 mL medium per 125 mL shake flask or 50 mL medium per 250 mL shake flask). 1 mL/L of Anti-Clumping agent was added to CD-CHO media to avoid clumping during culturing.

Note: MycoAlert™ mycoplasma detection kit can be used to test for any contamination according to the manufacturer’s instructions using Tecan Infinite™ F500 Luminescence.

2.2. Chemstress fingerprinting profiling of CHO cells

Chemstress fingerprinting profiling of CHO cells was performed as

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Fig. 2. Workflow of the directed evolution processes that demonstrates the use of the technology on CHO cell adaptation for different bioreactor chemical stressors.

Fig. 3. 80 phenotype stability trials for the Directed CHO process.
per the manufacturing protocol from Valitacell Ireland. Briefly, suspension CHO-K1 cells were cultured until reaching the cell density of $1 \times 10^7$ cells/ml. Cells were seeded into the ChemStress plates with a seeding density of 6000 cells per well. All the wells were filled with the seeding density of CHO cells and then incubated for a period of 72 h in a CO2 incubator. After 72 h of incubation, 10 μl of Presto Blue reagent was added to the 90 μL cell density in the well and incubated for 10 min at 37 °C. The 96-well microplate is measured for cell viability using fluorescence according to the manufacturer’s instructions using the Tecan Infinite™ F500 microplate reader (Fig. 1).

2.3. Manufacturing of EVO plates

We used Valitacell proprietary EVO plates for the directed evolution process. A Set of 2 EVO plates were printed with 21 stressors with 8 different concentrations in total. The stressors that were printed are involved in cell stress such as metabolic modulators, bioreactor toxins, cell cycle modulators, apoptosis mediators, epigenetic modulators, nutrient depletion, oxidative stress inducers, pH, and osmolarity stress (Table 1). The layout of the two EVO plates for the directed evolution process is proprietary to Valitacell (data not shown).

2.4. Directed evolution of suspension CHO cells

Suspension CHO-K1 cells are cultured in the Erlenmeyer flask for about 72 h at 5 % CO2, and at 37 °C until cell density reaches at least 10E6 cells. Note: Cell viability is measured using trypan blue staining as per the manufacturer’s instructions. 90 % cell viability is recommended.

![Fig. 4. Selected most stable sets 10 %) that are between the 1,2- 0.8 threshold range.](image)

![Fig. 5. Selected most unstable sets (10 %) that are between the 1,2- 0.8 threshold range.](image)
for directed evolution experiments. A seeding density of at least 40,000 cells/well in 300 μL CD-CHO medium with a concentration of at least 1.33E5 cells/ml is applied to seed cells into every well of the two deep well plates (DWP). 100 μL CD-CHO culture media are used to reconstitute the chemicals in the two EVO plates and an incubation period of 10 min is used to dissolve the chemicals at room temperature. Further, 100 μL of the reconstituted EVO chemicals were added to the 96 DWP containing 40,000 cells/well with 300 μL CD-CHO medium. 96 DWP are then sealed with Breathe-Easy® sealing membrane and incubated on an orbital shaker for 72 h at 37°C, 5% CO2, and 300 rpm using a clamp holder. On day 3 after 72 h of incubation, 90 μL of cells from DWP are added into the Black 96 well microplate with a clear bottom followed by 10 μL of Presto Blue reagent and incubated for 10 min at 37°C. The 96-well microplate is measured for cell viability using fluorescence according to the manufacturer’s instructions using the Tayan Infinite™ F500 microplate reader. For the second round of the directed evolution process, 200 μL CD-CHO media are added in each well of new 2 DWP, followed by 100 μL of cells from previous DWP into fresh DWP in fresh media, and finally, 100 μL of dissolved chemicals into fresh DWP with cells and media. This procedure was repeated for the 10 directed evolution cycles. The measured data are further analyzed together with all directed evolution cycles to demonstrate the phenotypic stability trial of CHO-K1 cell lines.

### 3. Results and discussion

We performed a directed evolution process in DWPs on suspension CHO-K1 cells using 21 stressors from Valitacell proprietary EVO plates as demonstrated in the workflow (Fig. 2). As expected during the first cycle of the directed evolution process where CHO-K1 cells were exposed to stressors, there has been a drop in cell viability across all conditions. There has been a concentration-dependent effect of stressors on cell viability with the lowest concentration of the stressor having the lowest effect on cell viability. However, as directed evolution cycles were repeated, there have been varying effects of stressors on the cell viability of suspension CHO cells (Fig. 3).

Previously, the Valitacell team demonstrated that [12] conventional stability trials and phenotype-based stability trials measure two different aspects of the cell lines. This paper focuses on performing a directed evolution-based phenotype stability trial for 21 stressors with 8 concentrations individually on 10 generations of CHO cells. Among 80 phenotype stability trials demonstrated in Fig. 4, we have identified stable sets of 10% close to control range 1 and are with phenotype stability trial range of 1.2 to 0.8 (Fig. 4). Similarly, an unstable set of 10% with a phenotype stability trial range of less than 0.8 (Fig. 5).

The phenotype stability trial was conducted by monitoring the cell viability of CHO cells incubated to stressors with different concentrations. Cell viability monitoring is performed for 10 cycles through presto blue reading. The prestoblue reading includes both absorbance and fluorescence measurement. The phenotype stability trial is compared to the control which is of value 1. To identify the most stable set, our stability cut-off is between 0.8 to 1.2, and the unstable set would be below 0.8 value. The rationale for choosing the stability cut-off of more than 0.8 is based on the correlation that 0.8 represents 80% viability in the presence of the stressor. However, these results will used further for stability trial-focused research.

### 4. Conclusion

This work focuses on the phenotype-based stability trial on CHO cells. To do so, we combined the Valitacell ChemStress™ EVO system with synthetic biology expertise at CSIR Pretoria to produce (1) a directed evolution process on their existing CHO hosts thereby enhancing the yield and reducing the cost of manufacturing. The uniqueness of our work is a) a first-of-kind miniaturized process that can simultaneously perform the directed evolution of CHO cells with 21 stressors with 8 different concentrations; b) a first-of-its-kind where the directed evolution process that can be done in deep-well with suspension cells. Thus, we demonstrated has reached a technology readiness level of 6 (TRL 6) (Table 2) with the requirement of additional validation for further progression in the TRL level.

This technology is currently established for CHO cell phenotype stability tests before scale-up. To perform similar studies with other mammalian cell lines, one needs to first identify chemical factors that would impact those cell lines in a bio-reactor setting. This is particularly crucial for the broad range of applicability of our directed evolution process. Furthermore, our direct evolution process has potential applications in emerging research areas such as synthetic biology [13,14], precision medicine [15], and nanobiotechnology [16] for industrial applications.

### Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Deepak B. Thimiri Govindaraj reports financial support was provided by International Centre for Genetic Engineering and Biotechnology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Author Contributions

DBTG Raj designed the project. DBTG Raj developed the methodology with inputs from Musasira N. DBTG Raj and Musasira N did all the experiments. DBTG Raj wrote the manuscript with inputs from Musasira N and Takundwa MM. This work was implemented as a Joint technology implementation between CSIR as a research council and Valitacell as an Industry partner. Valitacell owns the Intellectual property rights on ChemStress™ technology and associated technology development. CSIR implemented work as joint technology development and localization in South Africa.

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