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DEVELOPING AN ALTERNATE HOST FOR PRODUCTION OF BIOSIMILAR ANTI-EGFR MONOCLONAL ANTIBODY

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ABSTRACT

For the marketed version of anti-EGFR antibody, the production platform used by the innovator company is myeloma cells, SP2/0. This cell line is industrially less used and not well characterized. The cell density also could not be reached higher which eventually lead to lower expression levels. Typically an Immunoglobulin G1 molecule possess N-linked glycosylation site at Asn_{287} of Fc region, but the antibody in discussion possess a site Asn_{299} and also at Asn_{88} of Fab region. This additional glycosylation site increases complexity of molecule. In purview of getting high and stable expression of anti-EGFR antibody, CHO cells were used. CHO cells are well characterized with complete genome sequence information and are well accepted industrially. The CHO cells gave higher and stable expression when compared with SP2/0 cells.

KEY WORDS

Anti-EGFR antibody, cell line development, CHO cells, SP2/0 cells, cell line stability.

1. INTRODUCTION

Therapeutic monoclonal antibodies (mAbs) are as of today a well accepted class of therapeutics especially in the fields of oncology, immunology, and organ transplant, where the use of these targeted biologics has profoundly revolutionized treatments paradigms [1]. They are predominantly manufactured by mammalian cells in culture. Large-scale processes generally employ Chinese Hamster Ovary (CHO) cells as production vehicles, although other mammalian cell types, such as murine lymphoid cells (NS0, SP2/0), are also used. Mammalian cell hosts can correctly fold, assemble, and glycosylate mAb polypeptides. The latter is crucial, for example, in the case of recombinant mAbs that are designed to harness biological activities such as antibody-dependent cellular cytotoxicity (ADCC) and complement dependent cellular cytotoxicity (CDC) *in vivo* in addition to resistance to proteases, binding to monocyte Fc receptor, and determining circulatory half-life [2].

The use of mAbs has been exploited in treatment of various cancers in combination with either radiotherapy or chemotherapy. The dosage requirement of a mAb is larger than any other therapeutic proteins and hence high expressing cell lines are required to make the process economical. Achieving elevated expression yield is not the only criteria for claiming efficient process but, a balance between the quantity and quality needs to be finely tuned in order to get quality product matching biosimilarity aspects when compared with the approved innovator's product.

The biological characteristics are important as it configures the *in-vitro* and *in-vivo* potency of the molecule. These modifications / attributes are highly dependent on the production system, selected clonal cell population, homogeneity, clone stability and culture process, hence should be carefully studied and controlled before finalizing on the lead clone for the manufacturing of biosimilar therapeutic monoclonal antibody. The careful selection of single cell clones and optimization of cell culture conditions have been shown to impact the relative abundance of various antibody glycan structures. The enzymatic modification varies from cell to cell; right selection approach is important and crucial before identification of a Master Cell Bank (MCB) candidate clone.

One of the most discussed aspects in biologics is the glycosylation of produced molecule because it varies from cell lines to cell lines and process to process, but reasonably it is similar in the CHO, NS0 and SP2/0 cell lines. The point of concern is regarding glycosylation pattern distribution ratio (G0F, G1F and G2F) of the IgGs produced by NS0 and SP2/0 which is not similar to that of circulating human IgGs. In addition, these cells produce small amounts of murine-like glycans such as the addition of an extragalactose (α-Gal) to the terminal galactose and the insertion of N-glycolyneuraminic acid (NGNA) in place of N-Acetylneuraminic acid (NANA) [3], which have the potential to trigger an immune response. Because of these minor changes in glycosylation (e.g. NGNA) some clinical adverse events and anaphylactic shock have been reported for mAbs (such as Cetuximab) produced by cultivation of SP2/0 cells [4].

CHO cells being of rodent origin, the glycosylation pattern distribution ratio (G0F, G1F, G2F) of mAbs do not completely match with the circulating human IgG1. In addition, CHO cells produces a small amounts of non-human like glycan patterns, such as a 2-3 linked sialic acid residues that have the potential to be immunogenic [3]. But, these forms are present in very low proportions and mAbs produced from CHO cells have shown remarkable safe profiles in the clinic [5]. The choice of host cells for protein expression should be judiciously done and has a direct impact on product characteristics and maximum attainable yields. Protein folding and post-translational modifications conferred by the hosts dictate the pharmacokinetics and pharmacodynamic properties, and hence their solubility, stability, biological activity and residence time in humans. Product safety is another key aspect that must be considered in choosing host cells. The production host must not allow the propagation of any adventitious pathogenic agents that may eventually find their way into humans.

From an industrial perspective, the ability to adapt and grow cells in suspension instead of adherent cultures is highly desirable as it allows volumetric scalability and use of large stirred-tank bioreactors. Finally, the host cells must be amenable to genetic modifications allowing easy introduction of foreign DNA and expression of large amounts of desired protein. CHO cells are now there in industry for twenty five years and experience with it has demonstrated that, to a large extent, they possess many of these characteristics [6]. CHO cells have a proven track record for producing number of recombinant proteins and mAbs with glycoforms that are both compatible and bioactive in humans. One of the early concerns in recombinant protein production was that cultured mammalian cells were presumably derived through perturbation of oncogenes, and thus, can proliferate without the effects of senescence. However, CHO cells have been proven safe, with the value of products being generated considerably outweighing any associated risks.

In pursuit of establishing a stable high expressing cell line for anti-EGFR monoclonal antibody various vector constructs were designed with dual promoter system and different selection marker were evaluated. The dual promoter system facilitates expression of light chain and heavy chain of monoclonal antibody at similar molar concentration. Also, the integration of light chain and heavy chain sequence happen at the same region in the genome thereby making it convenient to establish and monitor the genetic stability and localization. The vector backbone used was pcDNA3.1(-) which is commercially available from Invitrogen, USA and is not covered under any patent terms.

The current work was undertaken to develop a cell line capable of expressing anti-EGFR mAb biosimilar to the innovator's product, using an alternate host cell platform (CHO cells), different from the one used by the innovator (SP2/0 cells). The expression profile and cell line stability of recombinant SP2/0 and CHO cells were studied.

2. MATERIALS AND METHODS

2.1 Gene sequence, Vectors and Reagents

The protein sequence encoding the light chain and heavy chain of anti-EGFR was determined by complete sequencing of gene by LC-MS/MS. The cDNA sequence coding the light chain and heavy chain was chemically synthesized and obtained from GENEART, Germany so that the sequences incorporate Signal peptide and other essential sequences (e.g., restriction sites and stop codon) at 5' and 3' end. The vector pcDNA3.1 (-) (Cat# V795-20) was obtained from Invitrogen, USA for research purpose. The transfection reagents used were from different vendors and the pool which gave expression of target protein was selected for further analysis.

2.2. Construction of Expression Vector

Two different expressions constructs with different selection markers, neomycine transferase (G418) and glutamine synthetase (GS) were employed for the selection in SP2/0 and CHO-S cells. The first vector named S8.1 contains GS gene while S8.3 expression constructs contains neomycine transferase as selection marker. Using pcDNA3.1 (-) as backbone the CMV promoter was PCR amplified from *Bgl*II and *Nhe*I sites and incorporating *Not*I and *Bam*HI site. The 900bp promoter was cloned in MCS of pcDNA3.1 (-) at *Not*I and *Bam*HI site resulting in a vector with two CMV promoter and two MCS sites. The light chain sequence was synthesized as an expression cassette of 872 nucleotides, of which the light chain sequence (mature protein) comprised 642bp and 164bp poly A and the signal sequence 60 nucleotides. The synthetic construct was amplified by PCR using forward and reverse primers synthesized from Sigma and cloned into MCS-1 of S8.1 and S8.3 expression vector at the *Nhe*I and *Xba*I site. The heavy chain sequence was synthesized as an expression cassette of 1444 nucleotides, of which the heavy chain sequence (mature protein) comprised 1347bp and the signal sequence 57 nucleotides. The synthetic construct was amplified by PCR using forward and reverse primers synthesized from Sigma and cloned into MCS-2 of S8.1 and S8.3 expression vector at the *Bam*HI and *Afl*II site.

The final expression construct was analyzed by RE analysis and DNA sequencing.

2.3. Bacterial transformation

Each ligation reaction (S8.1 or S8.3) were used for transformation of *E. coli* DH5-α electrocompetent cells. Cells were transformed by electroporation (Biorad-Pulsure,Voltage 2.5kv, Capacitance-25uF, & Resistance 200 ohm for 1 pulse and plated on SY agar (Hi-Media, India) plates containing 100 µg / mL ampicillin (Sigma,Cat# A1066) for positive selection.

Bacterial colonies obtained on SY ampicillin plate were picked up and checked using colony PCR

method. Two colonies giving PCR amplicon as expected was inoculated into 10 mL SY broth containing 100 µg / mL ampicillin and incubated overnight at 37°C with 200 rpm shaking speed. Subsequently, plasmid DNA was isolated from 2.0 mL culture using Promega wizard mini-kit and protocols (Cat# A1460). The DNA was eluted in 50 µL of elution buffer. The expression vectors (S8.1 & S8.3) were Restriction Enzyme analyzed to verify positive clones using *Nco*I enzyme. The digestion reactions were incubated at 37°C for ~2 h after which the samples were electrophoresed on 1 % agarose gel at 100 volts for 1.5 hr.

2.2 Cell culture

The SP2/0 Ag-14 cells (CRL-1581) was procured from ATCC, USA. The CHO-S (Cat# 1169-012) was procured to be used at R&D level from Invitrogen, USA.

The cells prior to transfection and following transfection before final selection were grown in DMEM media (Sigma, Cat #D5546) supplemented with 5% FBS (GIBCO Cat# 10099-133). The expression of protein of interest from SP2/0 and CHO cells were measured by ELISA. The shortlisted pools based on expression were diluted 100 cells / well to generate minipools. The top rated minipools were diluted at 1 cells/well to generate clonal population. The shortlisting of top 8 clones were carried out based on growth and productivity of clones. These shortlisted clones were adapted gradually in CD-CHO media (Invitrogen, Cat #10743) & supplements (Glutamine, HT & Pluronic F-68) used for evaluation of the top clones for primary cell bank preparation.

2.3 Enzyme Linked Immunosorbent Assay

The anti-human-IgG Fc specific monoclonal antibodies (Sigma, Cat # I6260) and anti-human IgG kappa specific HRP conjugated antibody (Sigma, Cat # A7164) were used for sandwich ELISA.

2.4 Transfection of CHO cells and mini-pool generation

2.4.1 DNA preparation

Both the recombinant plasmids (S8.1 and S8.3) were prepared using Pure Yield plasmid Midi-prep kit (Promega, Cat#A2492) after confirmation of the DNA sequences. The plasmids were linearized by *Pvu*I enzyme, which also disrupts ampicillin resistance marker gene. Digested DNA samples were purified using ethanol purification protocol [7]. The ethanol-purified DNA checked for purity.

2.4.2 Transfection of host cells

A day prior to transfection, cells were trypsinized using recombinant trypsin and seeded in 6-well TC plates at a density of 0.2 x 10^6 cells/mL. In two different sets of transfection using S8.1 and S8.3 expression vectors various transfection reagents were tried. The ratio of transfection reagent to DNA used was 3:1 with 1µg linearized DNA.

2.4.3 Mini-pool generation

The transient expression of target protein after 48 hours of transfection was evaluated by ELISA. Subsequently, cells were subjected to increasing concentrations of methyl sulfoxime (MSX), in case expression vector was S8.1 and of Geneticin G418 in case of expression vector S8.3. MSX concentration was started from 25 µM to 100 µM and G418 from 200, 500 and 750 µg/mL and maintained for 4 to 5 passages till cell growth was normalized. The expression was monitored at different intervals in SP2/0 and CHO pool. Minipools D41 was selected amongst 100 bulk pools based on productivity further clonal pool generation using manual limiting dilution technique.

2.5 Final clone selection

The final eight clones were selected from clones derived from mini-pool D41 based on cell growth and expression levels of different clones.

2.6 Adaptation in serum-protein free media and suspension

The eight clones were gradually adapted to chemically defined CHO media by step-wise dilution of serum containing media with serum free media. The cell growth and expression of target mAb was measured at each passage to monitor the clone behavior. Following this, the clones were adapted to suspension culture by inoculating cells at $2x10^6$ cells / mL in CD-CHO media and subjected to shaking conditions.

2.7 Cell Line Stability

The cell line stability was assessed under static and suspension conditions. The stability of eight selected clones was evaluated in presence of antibiotic and in absence of antibiotic. The top three clones stability was evaluated also under shaking conditions which to certain extend simulate bioreactor conditions.

3. RESULTS AND DISCUSSION

3.1 Construction and maintenance of dual vector system

The pcDNA3.1 (-) was modified to contain two CMV promoters for controlling expression of two genes independently named S8.3 vector. This vector was modified further where Neomycine transferase gene was replaced by GS gene resulting in S8.1 vector. The light chain sequence of ~0.8 Kb size and heavy chain sequence of ~1.5 kb of anti-EGFR mAb were cloned in independently in S8.1 and S8.3 vector. The final expression vector possessing light chain and heavy chain sequences are named as S8.1 and S8.3 expression vectors respectively (**Figure I).**

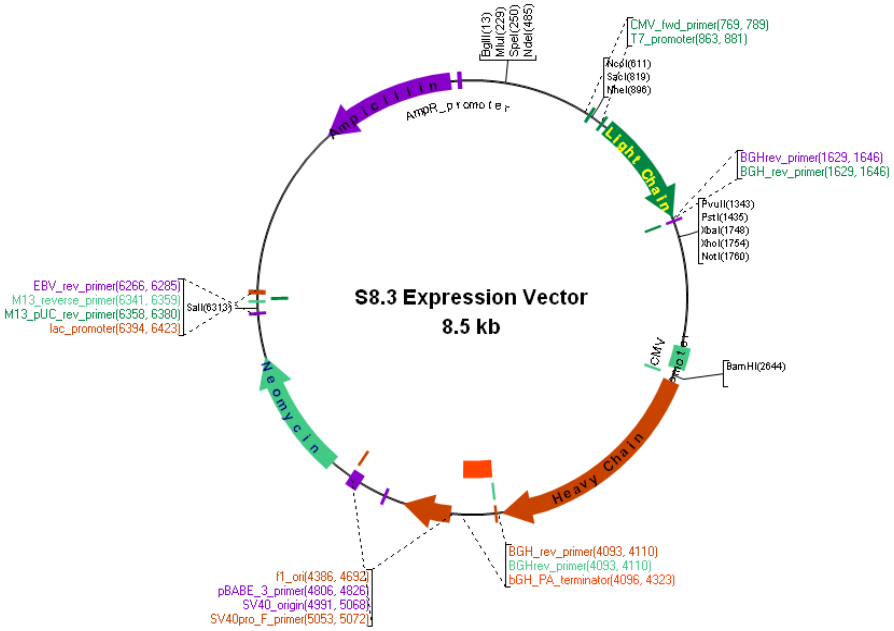

Figure I: Expression Vector designated as S8.3. The vector possesses two CMV promoters and two MCS. Expression vector S8.3 is similar to S8.3 except it has GS gene instead of Neomycin.

The vectors were transformed, maintained and amplified in *E. coli* DH5α cells.

Amongst several positive colonies observed after transformation, four colonies were screened for the correct orientation and authentication by digestion with *Nco*I enzyme **(Figure II).** One final colony was selected for the isolation of S8.3 expression vector to be transfected in CHO cells. Two clones from each combination were found positive and showed presence of desired DNA

fragments. Subsequently, these clones were verified by DNA sequencing and transfected in mammalian cell lines after linearizing using *PvuI* enzyme.

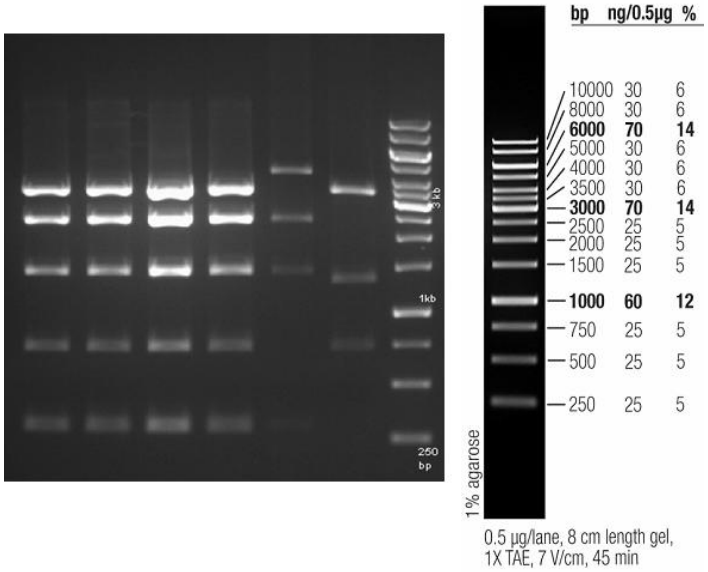

Figure II: RE Analysis of Final Expression vector from four different *E.coli* DH5 α clones (1% Agarose). Lane 1: Clone #5, Lane 2: Clone # 6, Lane 3: Clone # 7, Lane 4: Clone #8, Lane 5: pcDNA3.1 (-) with two CMV promoter, Lane 6: pcDNA3.1 (-) and Lane 7: 1kb GeneRuler. The inset shows the banding pattern of 1kb GeneRuler (Fermentas).

3.3. Comparison of expression of target protein in transfected SP2/0 and CHO cells.

The transfection of SP2/0 and CHO cells with S8.1 did not showed increase in expression when subjected to MSX, hence these transfectants were not pursued further (*data not shown*). The expression of target mAb was observed significantly higher when the CHO and SP2/0 cells were transfected with S8.1 expression vector. The expression in CHO was observed higher and stable than SP2/0 **(Figure IIIa and IIIb).** This could be due to more prevalence of gene silencing in SP2/0 or loss of recombinant construct from the cells.

3.4 Generation of 'Clonal Population'

CHO-s cells after transfection and based on expression analysis, Pool 'B' and 'D' were observed to be high producer. These pools were treated with gradual increase in antibiotic concentration. Pool 'D' was selected for further evaluation, based on growth characteristics and productivity. It was observed that the expression of target mAb do not increase after 200 µg / mL of G418. Hence 200 µg / mL G418 was continued for further selection and maintenance of the transfectants **(Figure IV).**

Pool 'D' gave highest expression of target mAb. The D41 minipool generated by diluting Pool 'D' was shortlisted based on expression yields and was further limit diluted to generate single cell clones. The single cell clones were selected and further analyzed for expression yields and product quality.

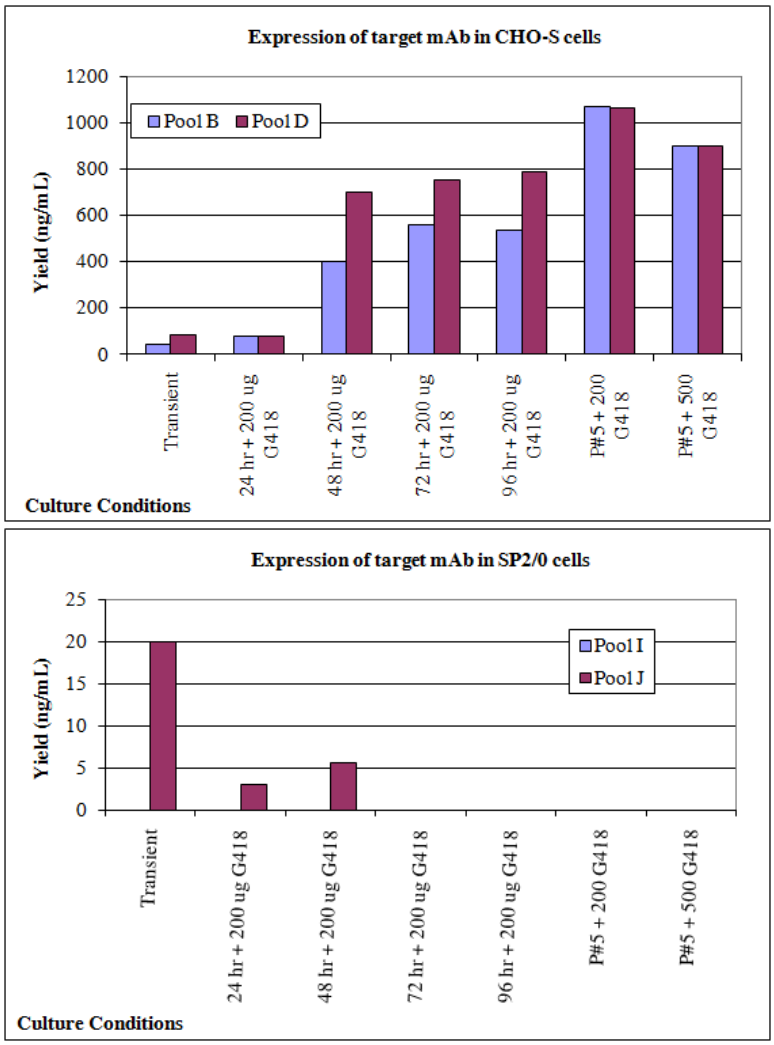

Figure III: Preliminary expression of target mAb after trasnfection analyzed by ELISA. Figure 3A: Expression of target mAb in CHO cells; Figure 3B: Expression of target mAb in SP2/0 cells.

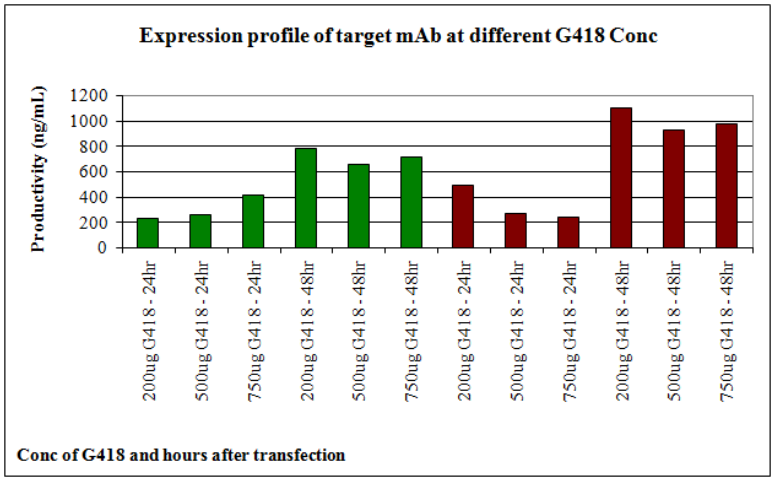

Figure IV: Expression profile of target mAb in CHO-S cells at different concentrations of G418. The green bars are for Pool 'B' and brown for Pool 'D'.

3.4. Selection of the best producer clone

Following limiting dilution of Minipool 'D41', six clones were observed to be high expressing under static conditions. When these six clones D41F109, D41G112, D41D116, D41C140, D41E213 and D41F284 were tested for expression under shaking conditions, D41F284 was observed to be highest producer followed by D41C140 and D41E213 **(Figure V)**. The average productivity of D41G277 was highest but the growth of cells was very sluggish and it didn't grow in subsequent passages. Hence, D41G277 clone was discontinued and D41F109, D41D116, D41C140, D41E213 and D41F284 were selected for further studies.

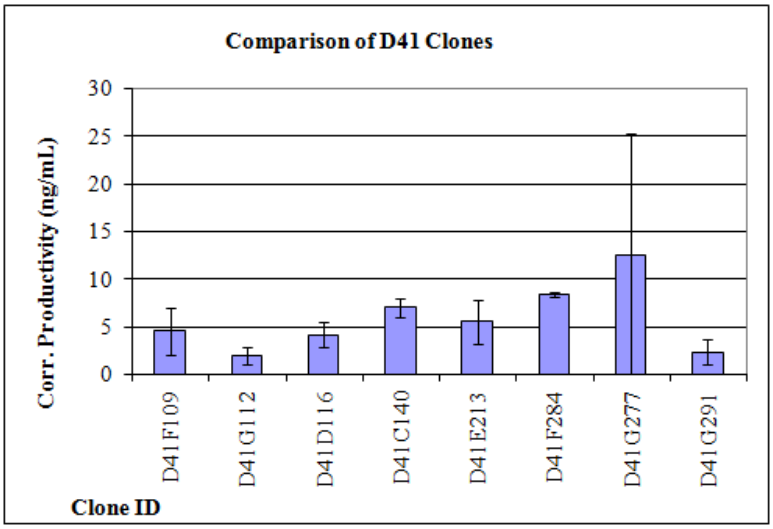

Figure V: Comparison of expression of target mAb in shake flask. The values plotted are replicates of three independent experiments.

3.4.1. Clone Stability Study

The clone stability is an important parameter to be monitored for the recombinant cell lines. Generally, the industrial production of recombinant therapeutic monoclonal antibodies are operated at >500L in order to meet patient's dose and market demand. The recombinant cell line generated for expressing target mAb should be genetically stable for sustained expression for at least 50 generations. This is because of the fact that in each passage about 3-4 generations are passed. Hence if we consider starting from master cell bank (MCB) preparation of ~200 vials then about 10 generations are utilized for MCB preparation and going till production bioreactor it will undergo ~20 passages. Further to this, if we start a production batch from 1 vial of MCB (1 mL) then it will start from 40 mL → 200 mL → 1L → 5L → 20L → 150L →500 L (18-20 generations) and growing cells for about 20 days for production hence another 15 generation, this all adds up to at least 45 generations. Considering this, the clone stability of top 6 clones D41F109, D41G112, D41D116, D41C140, D41E213 and D41F284 was monitored by *in-vitro* cell age for 76 days in a T25-flask in presence of antibiotic (Geneticin G418). The stability was determined by estimating recombinant protein production **(Figure VI).** At the end of 76 days culturing in presence of Geneticin-G418 loss of expression ~34%, 35% and 16% observed in clones D41D116, D41C140 and D41E213 respectively. This loss is not significant compared to other three clones where the loss is ~ 91%, 96%, and 74% in D41F109, D41G112 and D41F284 respectively. The loss of expression in latter 3 clones is in presence of selection pressure hence in absence of selection pressure

these would practically not produce anything when cultured for long time.

D41D116 and D41E213 clones showed a reduced expression on Day 39, however, in the next time point (Day 58) the productivity was more hence data of Day 39 is considered as an outlier.

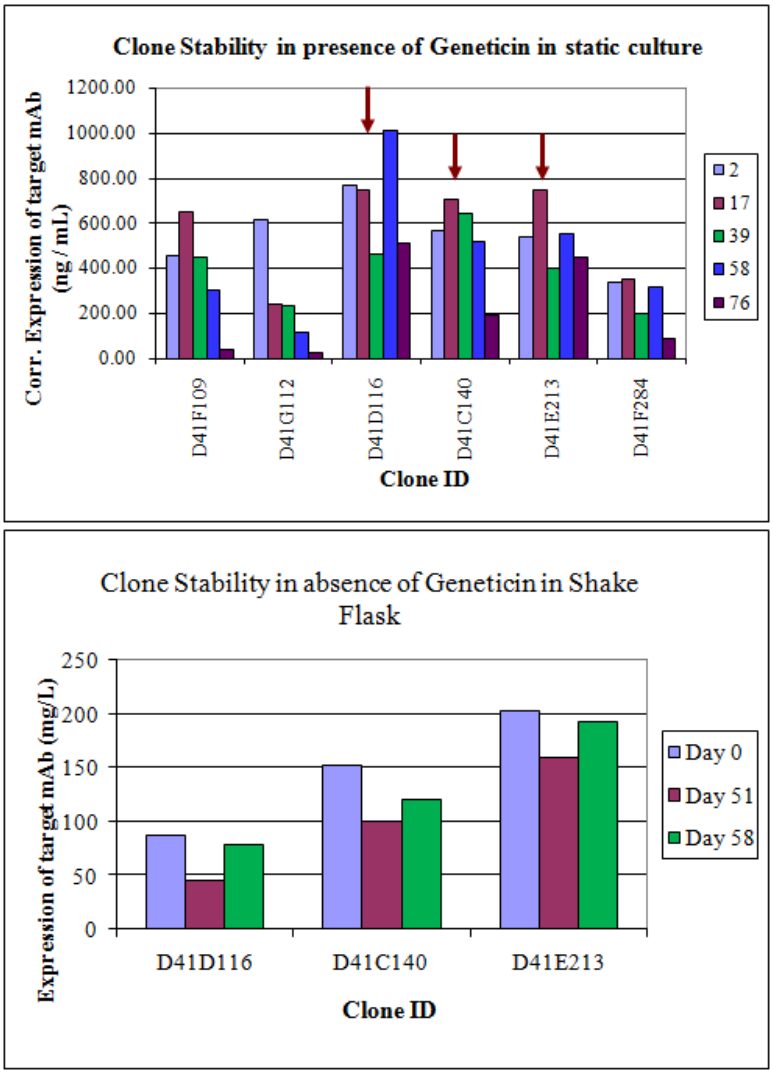

Figure VI: Clone Stability of recombinant CHO cells. A). Static culture in presence of Geneticin G418. Legends represents the days in culture; B) Shaking culture in absence of Geneticin .

The recombinant proteins are produced in bioreactor where agitation mode is used. Agitation is a physical parameter which could change the cell behavior hence there is need to prove the clone stability under shaking condition which mimics the bioreactor condition. D41D116, D41C140 and D41E213 were tested for clone stability in absence of selection pressure under shaking conditions to mimic culture conditions in bioreactor. The cells at different time points were subjected to fed-batch cultivation and the productivity at the end of batch was monitored. As shown in figure VI, the productivity of clone D41E213 was maximum and the loss of productivity was 5% when grown in absence of selection pressure for 48 days. In

clone D41D116 and D41C140 it was 11% & 9% respectively.

Hence, clone D41E213 is high producer with minimal loss in productivity in absence of antibiotic.

4. CONCLUSION

Two expression vectors with different selection markers was constructed and it was observed that no significant enhancement of expression was observed when glutamine synthetase was used as selection and amplification marker. The expression observed from the transfectants where expression vector with neomycin transferase as selection marker used was significantly higher.

Different transfection reagents/protocols were tried and SP2/0 was found to express target mAb at a low level. Moreover, the expression of anti-EGFR antibody declined with the passage and was short-lived. Hence an alternate alternate host system (CHO cell line) for expression of target mAb was done. The expression of target mAb was considerably higher and stable over a period of 50 generations.

The evaluation of product quality is important for proving the biosimilarity of the expressed product, especially in the case where the expression host is changed. The quality aspects of clones shortlisted based on growth and expression levels will be analyzed by employing a battery of analytical tests.

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