ADAPTATION OF CHOLESTEROL REQUIRING NS0 CELLS TO SERUM FREE CULTURE CONDITIONS

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ABSTRACT: Colorectal cancer is the third most common form of cancer and the second leading cause of cancer-related death in the Western world. The answers to such life threatening diseases and cancers are monoclonal antibodies (MAb's) which are widely used as therapeutic agents. World demand for currently approved MAb's is on the order of a few kilograms per year. However, new therapeutic MAb's are under development and require doses of several hundred milligrams to a gram over the course of therapy. Very often to cater for the special requirements for the growth of mammalian cells, serum is added to the cell culture medium. However, removal of serum from the cell culture medium is often carried out, especially if the end product is to be used for human consumption, in order to eliminate various disadvantages such as high physiological variability, high batch to batch variability, risk of contamination and high cost, and challenges posed in the downstream processing of the product. In this paper, the adaptation of cholesterol requiring NS0 cells to commercially available serum free media is presented.

ABSTRAK: Kanser kolorektum merupakan kanser ketiga paling umum dan kini berada di tempat kedua penyebab kematian berkaitan kanser di negara Barat. Jawapan kepada penyakit yang mengancam nyawa dan penyakit kanser adalah antibodi monoklon (*monoclonal antibodies ((MAb's*)) yang digunakan sebagai agen terapeutik. Permintaan dunia terhadap *MAb's* yang diluluskan adalah dalam bilangan beberapa kilogram setahun. Namun, terapeutik *MAb's* yang baru adalah di bawah penyelidikan dan memerlukan beberapa ratus dos milligram hingga satu gram dalam satu peringkat terapi. Sering kali untuk memenuhi permintaan terhadap tumbesaran sel mamalia, serum dicampurkan dengan sel kultur perantara. Walaupun begitu, pemindahan serum dari sel kultur perantara sering dilakukan, terutamanya jika produk akhir digunakan untuk kegunaan manusia; untuk mengurangkan pelbagai kelemahan seperti kebolehubahan psikologi yang tinggi, kebolehubahan yang tinggi daripada satu kumpulan ke satu kumpulan lain, risiko pencemaran, kos yang tinggi, dan cabaran mendatang dalam pemprosesan produk. Dalam perbentangan ini, kolestrol yang diubah memerlukan sel *NS0* yang dikomersilkan dengan serum bebas perantara.

KEYWORDS: *NS0; serum-free; monoclonal antibody; growth*

1. INTRODUCTION

The usage of monoclonal antibodies (mAbs) as therapeutic agents has seen a rapid increase during the past decade or so and thus there are several approved

antibody based medicinal products in the market today. The prime advantage of mAbs as therapeutic agents is their high target specificity, resulting in a low side effect profile. Monoclonal antibodies can also be engineered to have additional biological activity beyond binding of the antibody to its antigen.

Due to the high the therapeutic potential, the process development and production of monoclonal antibodies is being pursued by biopharmaceutical companies globally. Monoclonal antibodies are primarily manufactured in genetically engineered mammalian cell lines such as NS0, CHO, etc. NS0, a murine myeloma cell line, has been adopted by a number of biotechnology companies for the expression of therapeutic antibodies and has proven to be effective and easily lent itself to process optimization efforts [1-3, 9, 15, 16]. Thus, the importance of mammalian cell culture for the production of special biopharmaceuticals and biodiagnostics not only in research but also on clinical diagnostics and therapy has increased exponentially. The products which are produced for direct application to humans must be of extremely high purity and constant quality, and at the same time, the production processes should be economically optimized and efficient [6].

Very often to cater for the special requirements for the growth of mammalian cells, serum is added to the cell culture medium. This is because serum contains plethora of proteins, peptides, growth factors, hormones and other components which may be of importance for the cells to be cultivated and may not be present in the serum free media at all [14]. Due to these properties, removal of serum from the culture medium is a daunting task. However, removal of serum from the cell culture medium is often carried out in order to eliminate various disadvantages posed by its usage which include high physiological variability, high batch to batch variability, risk of contamination and high cost, and challenges posed in the downstream processing of the product, etc. [5, 6, 8, 10, 13, 14]. It should however be noted that the development of a serum-free culture processes has been particularly challenging for NS0 cells due to their cholesterol autotrophy [10-12].

In order to adapt the cells to Serum free media, generally two approaches are common: Direct Adaptation and Indirect (Sequential) Adaptation. In the former case, cells are directly cultured from a growth medium containing serum to a serum free medium, whereas in the latter case, the concentration of the growth medium containing serum is slowly reduced and replaced with serum free medium [7].

2. METHODOLODY

2.1 Cell Culture Media and Supplements

Three commercially available serum free media, HyQ CDM4NS0 (Thermo-Scientific, Product # SH30579.02), Excell-NS0(Sigma Aldrich, Product # 14650C) EX-CELL 610-HSF (Sigma Aldrich, Product # 14650C) and combination of DMEM and HyQCDM4NS0 in certain ratio were tested for the direct adaptation of the cells to serum free conditions.

The supplements that were used included ultra low IgG FBS (Gibco, Product # 16250-078) in a concentration range of 1-5% (v/v) and Synthechol (SigmaAldrich, Product #) in a concentration range of 0.2-0.4%(v/v). Also, L-glutamine, 2 mM (Sigma, Product # G8540) was supplemented to the medium.

2.2 Cell Line and Routine Culture Conditions

The NS0 wild type cell line was obtained from InnoBiologics Sdn. Bhd., with the courtesy of Dr. Cristina Mateo, Center for Molecular Immunology (CIM, Havana, Cuba). These cells secrete humanized anti- CEA (carcino-embryogenic antigen) and are cholesterol auxotrophs. The cell line was maintained in cryo vials until usage. Following vial thaw, cells were routinely grown in static culture using 25 cm^2 T-flasks containing 10 ml of DMEM medium supplemented with 2 mM L-glutamine and 5% ultra low IgG FBS. The cell cultures were sequentially adapted to 1% ultra low IgG FBS prior to carrying out any direct adaption to serum free media. The cultures incubated at 37°C with a 5% CO2. Every 24-96 hours, when the cells had grown to a density of about 1 x 10^6 cells/ml or more, the culture was passaged by dilution with fresh growth medium to approximately 0.3-0.5 x 10^6 cells/ml. In the presence of serum most of the cells grew loosely attached to the plastic flask and the adherent cells were easily removed by firmly tapping the side of the flask.
For both direct and sequential adaptation, cells were seeded form the current growth medium at a seeding density of 0.5 x 10^6 cells/ml.

2.3 Analytical Methods

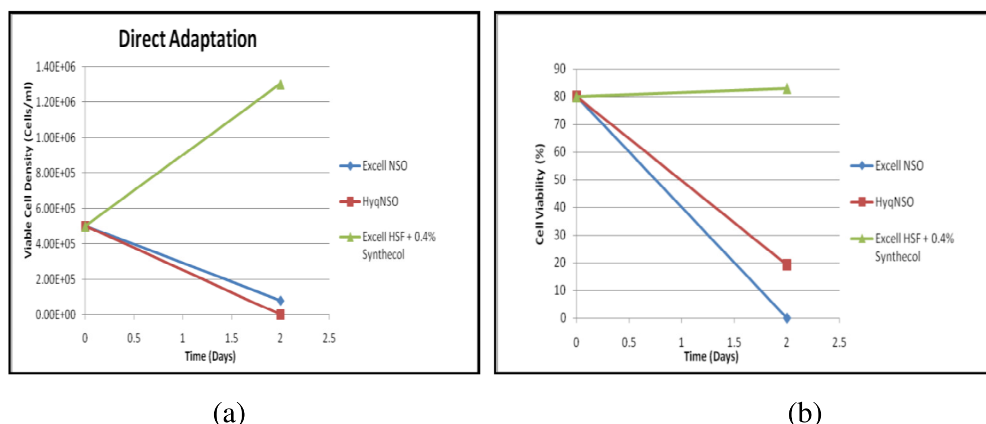
Viable cell density (VCD) and viability were determined by the tryphan blue exclusion method using a haematocytometer. Prior to cell counting, samples were diluted 2 to 10-fold with 0.4% trypan blue solution (Gibco, Prdouct # 15250-061) depending on the cell density. In serum-containing static culture, the cells were first dislodged by tapping the side of the flask.

An enzyme-linked immunosorption assay (ELISA) was employed to determine the titer of the monoclonal anti-carcinoembruogenic antibody. Cells were centrifuged for 5 min at 6000 rpm with a Minispin plus microcentrifuge (Eppendorf AG, Hamburg, Germany) prior to the antibody assay. Human IgG, whole molecule, unconjugated (Pierce, Product # 31154) was used as a standard. First, 100 µl capture antibody solution with a dilution of 1:1000 (anti-human IgG-Fc specific, produced in goat; Sigma, Product # I2136) was placed in a 96-well microliter plate and incubated at 2-8°C overnight. Next, after rinsing with 1x200 µl washing buffer (0.05% Tween-20 in 0.9% NaCl), 200 µl of blocking buffer (0.01% w/v BSA and 0.02% v/v Tween-20 in 1XPBS) was added and incubated for another 2 hours at 37°C followed by washing 4x with washing buffer. A 100 µl sample or standard solution was added to each well and incubated for 2 hours at 37°C. After rinsing with 4x200 µl washing buffer, 100 µl labeled secondary antibody, 1:2500 dilution (anti-human gamma chain specific peroxidase conjugate, produced in goat; Sigma, Product # A6029) was added and incubated for another 2 hours at 37°C. The plate was then rinsed with 4x200 µl washing buffer. Then, 100 µl of enzyme substrate working solution (ABTS tablets, Roche, Product # 11 112 442 001, dissolved in ABTS buffer, Roche, Product # 11 112 597 001) was added and incubated for 1 hour in the dark. Absorbance was measured at a wavelength of 405 nm via a kinetic microplate reader (Bio-Tek Instruments Inc., Highland Park, Winooski). Samples were diluted 1000 to 10,000-fold with blocking buffer prior to assay depending on the antibody concentrations.

3. RESULTS AND DISCUSSION

3.1 Direct Adaptation

Out of three media tested for the direct adaption of the NS0 cells, only one of them (EXCELL 610HSF) showed potential in supporting the growth of the cells (Fig. 1). Only cells in Excell 610HSF showed growth after 48 hours of the subculture with a Viable cell density of 1.30×10^6 cell/ml and a cell viability of 82.96%. In comparison, cells in HyQ CDM4NS0 dropped down to a viable cell density of 8.0×10^4 cells/ml compared to 5.0×10^5 at the beginning of the culture and a cell viability of only 19.23%. Also the result with Excell NS0 was not much different from HyQ CDM4NS0, in the sense that all cells were dead after 48 hours.

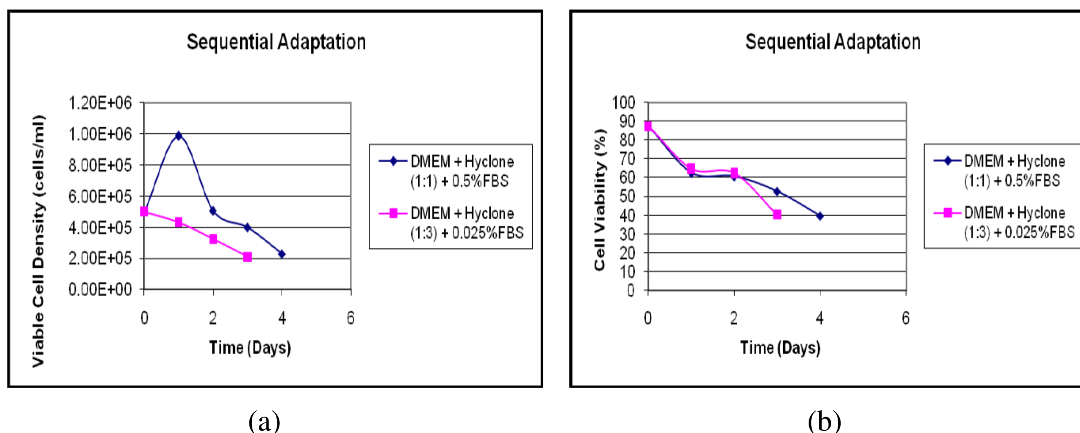


(a) (b)

Fig. 1: (a) Direct adaptation in T-25 flask, Viable Cell density (cell/ml) versus Time
(b) Direct adaptation in T-25 flask, Cell Viabilty (%) versus Time (days).

3.2 Indirect Adaptation

For sequential adaptation, the cells were first grown in DMEM + Hyclone (1:1) + 1% FBS. After three passages, cells were sub-cultured again to DMEM + Hyclone (1:1) + 0.5% FBS and then to DMEM + Hyclone (1:3) + 0.025% FBS. As is evident from the batch results in Fig. 2 (a and b) the cells don't grow well and the peak cell density reached is below 1×10^6 cells. Also, the cell viability just keeps dropping since day one.

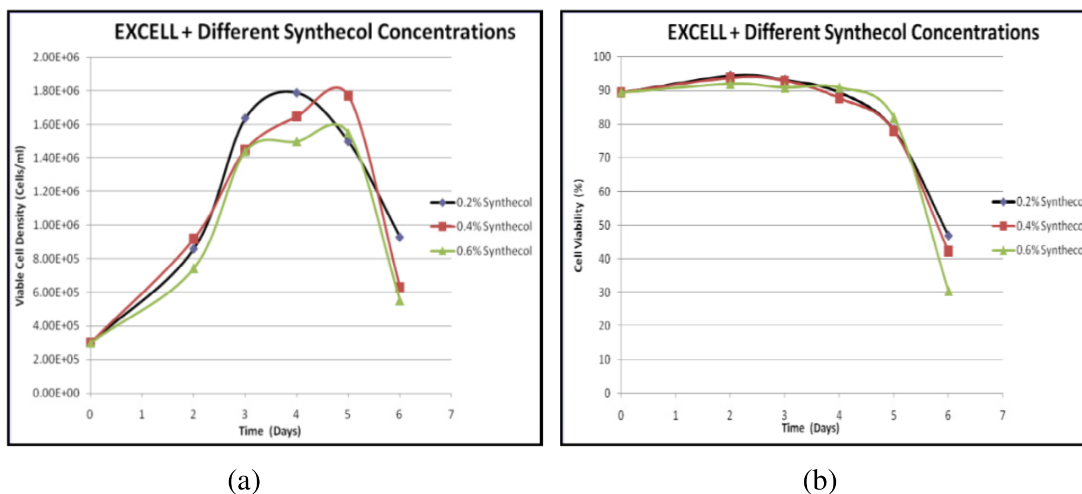


(a) (b)

Fig.2: (a) Sequential Adaptation in T-25 flask, Viable Cell density (cell/ml) against Time (Days).
(b) Sequential Adaptation in T-25 flask, Cell Viabilty (%) against Time (days).

3.3 Effect of SyntheChol on NS0 Cell Growth and Productivity

Since Excell610HSF + 0.4% Synthechol supported the serum free growth of NS0 cells, further investigation was carried out for determining the optimal concentration of Synthechol supplement for the media. According to the manufacturer's recommendation (Sigma-Aldrich) three different concentrations of Synthechol were tried and the culture was seeded at 3.0 x 10^5 cells/ml (Fig. 3).



(a) (b)

Fig. 3: (a) Excell + Different Synthechol Concentration in T-25 flask, Viable Cell density (cell/ml) versus Time (Days).
(b) Excell + Different Synthechol Concentration in T-25 flask, Cell Viabilty (%) versus Time (days).

As is evident for the Fig. 3 (a and b) above, the maximum cell density was achieved using 0.4% Synthechol concentration on day 5. Although the cell viability using 0.4% Synthechol was not as high in comparison to 0.2% and 0.6% Synthechol supplement concentration, but the value was above 90% and remained throughout until the peak cell concentration was observed on day 5.

In terms for antibody titer, the culture with 0.2% Synthechol concentration produced a cumulative of 31.18 µg/ml in comparison to 29.10 µg/ml using 0.4% Synthechol and 27.46 µg/ml using 0.6% Synthechol, indicating that Synthechol concentration of 0.2% v/v is the best of the three for production secretion (Fig. 4).

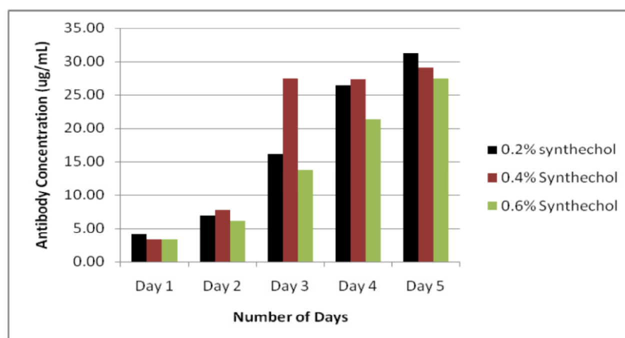


Fig. 4: Monoclonal anti-CEA antibody concentration using different Synthechol concentrations.

4. CONCLUSION

NS0 wild type cell line was successfully adapted to serum free culture conditions. Due to cholesterol auxotrophy, adaptation of NS0 cells to serum free conditions enabled to determine the optimum concentration of Cholesterol supplement (Synthechol) for monoclonal antibody production.

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