ADAPTATION OF ANTI-CEA HYBRIDOMA CELL LINE TO SERUM-FREE CULTURE MEDIUM BY USING SLOW ADAPTATION TECHNIQUE

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Thesis submitted in fulfillment of the requirements
for the award of the degree of
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ABSTRACT

In animal cell culture, serum is normally added in the cell culture medium to provide cells growth, regulates cell membrane permeability and also serves as a carrier for lipids, enzymes, micronutrients, and trace elements into the cell. However, serum has many drawbacks such as batch to batch variability, high price, interference with the purification of recombinant product, and the possibility of viral contamination. To address those threats, removal of serum from the culture medium is often carried out. In this study, a hybridoma cell line producing humanized antibody against the carcinoembryonic antigen or anti-CEA, will be adapted in a serum-free culture medium (SFM) by the slow adaptation technique. Cells were cultured in culture medium that is composed of serum supplemented medium (SSM) and SFM at varying ratios (100:0; 95:5; 90:10; 85:15 and 80:20). The technique was assessed by determining the cell density, cell viability as well as the glucose uptake and lactate release of the cells. Results indicated that the cell concentration was significantly reduced as the concentration of serum was decreased (when SSM: SFM ratio increases), and therefore the technique was considered as not compatible for adapting the specific cell line in SFM.

ABSTRAK

Dalam kultur sel haiwan, serum biasanya ditambah dalam medium kultur sel untuk pertumbuhan sel, mengawal kebolehtelapan membran sel dan juga berfungsi sebagai pembawa untuk lipid, enzim, mikronutrien, dan unsur surih ke dalam sel. Walau bagaimanapun, serum mempunyai banyak kelemahan seperti kumpulan kepada kumpulan variasi, mahal, gangguan dengan ketulian produk rekombinan, dan kemungkinan untuk dicemar oleh virus. Oleh itu, untuk mengelakkan masalah-masalah tersebut, penyingkiran serum dari medium kultur yang sering dijalankan. Dalam kajian ini, sel hybridoma menghasilkan antibodi terhadap antigen carcinoembryonic atau anti-CEA, akan disesuaikan dalam medium tanpa serum dengan teknik penyesuaian yang perlahan. Sel dikulturkan dalam medium yang terdiri daripada serum sederhana ditambah dan medium tanpa serum pada nisbah yang berbeza-beza (100: 0; 95: 5; 90:10; 85:15 dan 80:20). Teknik ini telah diuji dengan menentukan ketumpatan sel, sel daya maju serta pengambilan glukosa dan laktat pembebasan sel-sel. Hasil menunjukkan kepekatan sel telah dikurangkan dengan ketara sebagai kepekatan serum telah menurun (apabila SSM: SFM nisbah kenaikan), dan oleh itu teknik yang telah dianggap sebagai tidak serasi untuk menyesuaikan sel tertentu dalam medium tanpa serum.

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LIST OF ABBREVIATION

ADF	Animal-Derived Component Free
CD	Chemically Defined
CEA	Carcinoembryonic antigen
CHO	Chinese Hamster Ovary
CO_2	Carbon Dioxide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factors
HCl	Hydrochloric acid
Ig	Immunoglobulin
mAb	Monoclonal antibody
M-CSF	Macrophage Colony-Stimulating Factor
NaOH	Sodium hydroxide
NGF	Nerve Growth Factor
PFM	Protein-Free Medium
SFM	Serum-Free Medium
SSM	Serum-Supplemented Medium

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

The development of cell culture of mammalian cells has been studied from past 50 years (S.Ozturk, 2006). Basically, the cell culture is a process of removing cells from animal tissue and will grow in the nutrients and growth factors medium (Sennott R.Stephen, 2004). The first attempt to culture animal cells was to grow them in a biological fluid like serum or tissue extracts including plasma, serum, lymph, chicken embryos leaching solution (Yang & Xiong, 2012). Then, it was attempted to culture in defined media. Later on, many new formulations were developed as new cell lines became available including the Dulbecco's Modification of Eagle's Medium (DMEM). Then many varieties of serum-free formulations were designed upon development on cell metabolism and growth factor requirement (S.Ozturk, 2006).

Serum provides a broad spectrum of macromolecules, proteins, attachment and spreading factors, low molecular weight nutrients, hormones, and growth factors (Even et. al, 2006). The most commonly used animal serum supplements are Fetal Bovine Serum (FBS) and Fetal Calf Serum (FCS) (Even et. al, 2006). The benefits of serum removal are decreased variability in qualitative and quantitative culture medium composition, and reduced risks of microbial contamination such as mycoplasma, viruses, and prions (Brunner et al., 2010).

Then, the formulation of Serum-Free Media has been developed for many cell lines including hybridoma and Chinese Hamster Ovary (CHO) cells. One of the main advantages of the control over growth promoting activity afforded by serum-free media is the ability to make a medium selective for a particular cell type (Freshney, 2005).

1.2 Problem Statement

In animal cell culture, serum is an essential supplement. However, serum has many disadvantages such as batch variability, inadequate supply, interference with the purification of recombinant product, the possibility of viral contamination, high price and ethical issue (Liu & Chang, 2006).

Physiological variability occurs when major constituents of serum such as albumin and transferrin, as well as minor components e.g. amino acids, nucleotides, sugars, peptide growth factors, hormones, minerals and lipids varies from batch to batch. In the downstream processing, the presence of serum creates a major impediment to product purification and may even limit the pharmaceutical acknowledgment of the product (Even et al., 2006).

Not only that, serum is often contaminated with viruses, which may be harmful to cell culture but represents an additional unknown factor outside the operator's control. Viral infection remains a problem even improvement in serum sterilization techniques has been invented (Even et al., 2006).

High cost is another major drawback of serum supplementation. Lastly, fetal bovine serum (FBS) production methods have raised concerns about the welfare of the animals. FBS is harvested from bovine foetuses taken from pregnant cows during slaughter and is generally acquired by means of cardiac puncture without anesthesia (Even et al., 2006). So in order to abolish these drawbacks, removal of serum from the culture medium is conducted (Raja et al., 2011).

1.3 Research Objectives

This research aims:

1.3.1 To adapt anti-CEA hybridoma cells from serum- supplemented medium (SSM) to serum-free culture medium (SFM) using slow adaptation.

1.3.2 To determine the growth profile, morphology and metabolic activities of anti-CEA hybridoma cells in different medium compositions

1.4 Scope of Research

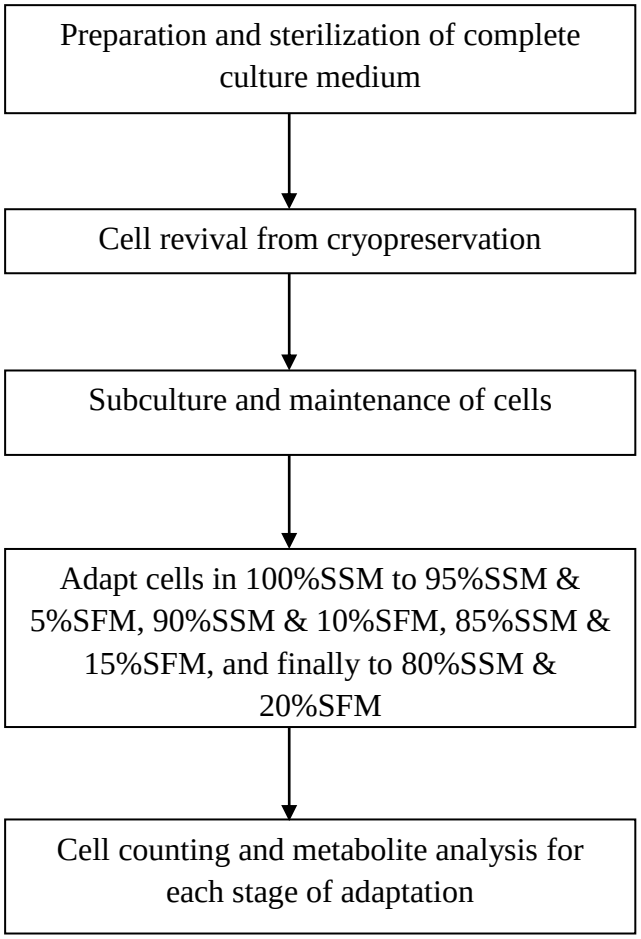
The scopes of this research are:

i. Slowly adapt cells cultured in serum- supplemented medium (SSM) to serum-free culture medium (SFM) in T-flasks at 37°C in a 5% CO_2 humidified atmosphere

ii. Determine the effectiveness of this adaptation technique by measuring the cell density, cell viability, glucose uptake and lactate production of the cells

1.5 Research methodology

In order to achieve the aims of this research, the following steps are performed:



CHAPTER 2

LITERATURE REVIEW

2.1 Cell culture

In cell culture the cells were removed from animal tissue or whole animal will continue to grow if supplied with nutrients and growth factors. This process is called cell culture. It happens *in vitro* (‘in glass’) as opposed to *in vivo* (‘in life') (M.Butler, 2004). Cell cultures are derived from dispersed cells taken from original tissue and disaggregated by enzymatic, mechanical, or chemical means (Freshney, 2005). Many cell types can be routinely proliferated and cryopreserved allowing consistent, characterised, contaminant free cells to be used as both a research and diagnostic tool. The experimental work of Hay flick and Moorhead in the 1960s defined the finite life span of mammalian cells. Molecular biologists explained this in terms of the gradual reduction of the telomeres at the end of the chromosomes (M.Butler, 2004).

Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrates (Sato, 1980). Primary cell cultures can be established from whole animal embryos, or from selected tissues from embryos, newborn animals, or adult animals of almost any species. The most commonly used cell cultures in virology are derived from primates, including humans and monkeys; rodents, including hamsters, rats, and mice; and birds, most notably chickens (M.Butler, 2004).

2.2 Applications of animal cell culture

M.Butler (2004) has listed several applications for animal cell culture. One of the applications is the study the normal physiology or biochemistry of cells. For example, metabolic pathways can be investigated by applying radioactively labeled substrates and subsequently looking at products. Next application is to test the effects of compounds on specific cell types. Such compounds may be metabolites, hormones or growth factors. Similarly, potentially toxic or mutagenic compounds may be evaluated in cell culture. Thirdly is to synthesize artificial tissue by combining specific cell types in sequence. This has considerable potential for production of artificial skin for use in treatment of burns. Last application is to produce valuable biological products like monoclonal antibodies and glycoproteins from large-scale cell cultures.

2.3 Type of cells

2.3.1 Adherent

Adherent cells which are also known as anchorage-dependent cells require attachment to a substratum for their proliferation. They are generally subject to contact inhibition, such that they grow as an adherent monolayer and stop dividing when they reach a density that they touch each other. Most cells with exception of mature hematopoietic cells and transformed cells grow in this way (Kumar, 2008). In the laboratory scale the substratum can be provided by the solid surface of Petri dishes, T-flasks, or Roux bottles which are made of specially treated glass or plastic. The interaction between the cell membrane and the growth surface is critical and involves a combination of electrostatic attraction and van der Waal's forces (Butler, 2004). Cells bind to surface through specific cell surface receptors to attachment proteins absorbed to the surfaces (Kumar, 2008).

2.3.2 Non-adherent (suspension)

Suspension cultures are gained from cells that can stay alive and proliferate without attachment to the substratum which is the anchorage independent. Examples of this cell type are transformed cell lines, cells from malignant tumors and cells of hematopoietic origin (Freshney, 2005). These cell lines do not produce attachment factors and stay in suspension either as single cells or as clumps of cells.

Lymphoblastoids and many other tumour cell lines do not require a surface on which to grow and therefore do not require proteases such as trypsin for sub-culturing (O'Connor Lorraine, 2006). Sub-culturing or passaging is done when cells stop growing in culture and by inoculating cells into fresh medium new cultures can be established. Subculture cells within few days are important to make sure it grows continually in a new culture. The viability of cells will decrease if they left for longer time before subculture. (Sennott R.Stephen, 2004).

The main disadvantage of suspension culture lies in its technique of sub-culturing which is by dilution. During growth, cells produce metabolic by-products, which become toxic if allowed to accumulate in the medium. Initially, sufficient waste metabolites will be removed by diluting cell cultures, allowing growth to continue but the viability of the cells will gradually decline after a few subcultures (O'Connor Lorraine, 2006).

2.4 Cell growth phases

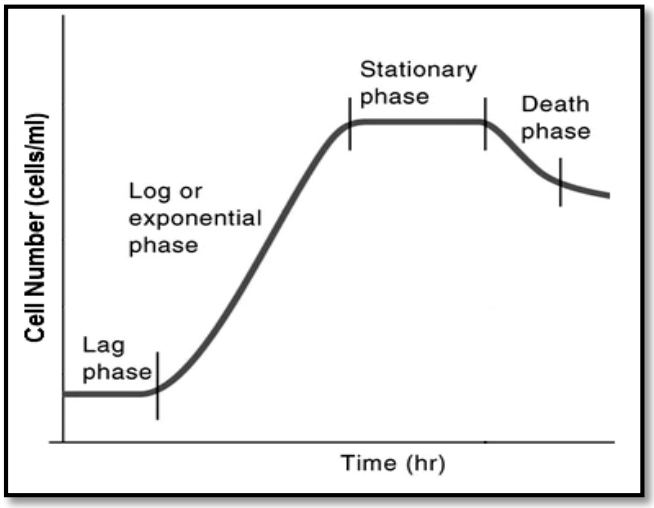


Figure 1: Typical Growth Phases of Cells

The lag phase is an early phase in which there is no clear increment in cell concentration. This phase is connected with the cellular synthesis of development factors which might be required to achieve a critical concentration before development happens. The culture medium formulation, initial concentration and state of the cells can affect the length of this phase. In the exponential phase, the cells have adjusted to their fresh environment. After the adaptation period (lag phase), cells can multiply rapidly, and cell mass and cell number density increase exponentially with time. During

balanced growth, the net specific growth rate, μnet determined from either cell number or cell mass would be the same. The doubling time also can be calculated based on cell numbers and the net specific rate of replication. The stationary phase happens when there is no additional increase in cell concentration. At this phase, the death rate is same as the growth rate. This is due to depletion of nutrients where it cannot bear additional cell growth. Then, there is might have a lack of growth space. So the growth of cells could stop when particular monolayer covers the available substratum. The decline phase takes place due to cell death. A larger difference between total and viable cell counts indicates decline phase of culture. The cell concentration decreases due to cell lyses and intracellular metabolites that released into the growth media (Sennott R.Stephen, 2004).

2.5 Hybridomas

Hybridomas can be developed in suspension in large bioreactors for the production of larger amounts of monoclonal antibodies. The process of cell fusion will bring about a heterogeneous population of cells that will contain unfused parental cells, lysed cells, and essential hybrid cells. At this stage, the selection of the cell is crucial so that the hybrid cells can be disengaged from the mixture. For hybridomas, there are two significant stages of cell selection. First is segregation of hybrid cells from parental cells and second is the selection of antibody-secreting cells within the hybrid cell population (Sennott R.Stephen, 2004).

The specific protein fraction of blood called the gammaglobulin or the immunoglobulin fraction is found in antibodies. B-lymphocyte is able to produce one type of antibody in response to a particular antigen which interacts with a cell surface receptor. A particular antibody will be produced in an animal by injecting the corresponding antigen (Sennott R.Stephen, 2004). The choice of producing antibody hybridoma clones is generally clear or straightforward (Vetterlein, 1989).

Carcinoembryonic antigen (CEA) is a particular marker for colorectal cancer and has been broadly acknowledged as a diagnostic adjunct to colorectal growth (Lin et al., 2016).A procedure for producing monoclonal antibodies particular to Carcinoembryonic Antigen (CEA) which inoculating mammal with a first CEA to deliver cells equipped for creating antibodies (Webb & Schumm, 2003). One way to produce monoclonal

antibody to CEA is by inoculating the mammal with CEA to sensitize the immune system of the mammal and therefore producing the anti-CEAs (Webb & Schumm, 2003).

2.6 Monoclonal antibodies

The expression "monoclonal" shows that the immune antibody is of a single type. This will attach to just one antigen. Due to their high specificity in recognizing selected proteins, the antibodies have a scope of applications. Thus, it is used for determination and testing in the application such as blood typing, pregnancy testing, and the recognition of infection or for the detection of contaminants in food (Sennott R.Stephen, 2004).

Furthermore, the advantages and benefits of hybridoma technology are the monoclonal antibodies is homogeneous and predictable because it is gain from one isolated clone, pure antibodies can be made to impure or unknown antigens, unlimited amounts of antibody can be obtained, and for an indefinite period and lastly the technology is cheap and took less time than conventional immunization (Jerry W.Shay, 1985).

The therapeutic use of monoclonal antibodies has taken some time because of a range of side-effects connected with undesirable immune responses in human of murine-derived antibodies. The situation is quickly changing with the capacity to create humanized or fully human antibodies. This has empowered the approval of monoclonal antibodies for a scope of treatments including transplantation, cancer, infectious disease, cardiovascular disease and inflammation (Sennott R.Stephen, 2004).

When a complex antigen, for example, a protein is brought into an animal, it contains numerous antigenic determinants. The outcome is that many B cells will be stimulated to deliver antibodies directed against the antigen. This happens even when very much purified antigen is used as an immunizing agent. Sera that obtained are heterogeneous which is immunized from animals. By a proper adsorption, this can be reduced, however, it not dispensed. Other than that, the major troubles with conventional immunization are unpredictability of the immune response, heterogeneity of even extremely specific antibodies, supply is frequently restricted, identical antibody

cannot be made in a new animal or from a second or third bleeding of the same animal because every bleeding is a new reagent and the process is quite expensive and take time (Jerry W. Shay, 1985).

The therapeutic use of monoclonal antibodies has taken some time because of a range of side-effects connected with undesirable immune responses in human of murine-derived antibodies. The situation is quickly changing with the capacity to create humanized or fully human antibodies. This has empowered the approval of monoclonal antibodies for a scope of treatments including transplantation, cancer, infectious disease, cardiovascular disease and inflammation (Sennott R.Stephen, 2004).

2.7 Culture medium

The culture medium must contain nutrients necessary to the production of new cells and substrates for accomplishing cell metabolism, besides compounds allowing physiological and catalytic functions, or which act as cofactors. To maintain the conditions found in the original tissue from which a particular cell originated, it is necessary for a culture medium to contain few supplements such as inorganic salts, sugars, amino acids, vitamins, lipids, organic acids, proteins, hormones, carbon and nitrogen sources, micronutrients (organic ions and minerals), antibiotics and water as well as cell-specific substances (Freshney, 2005).

There are few types of synthetic media available for the cell culturing such as chemically defined media, animal-derived component free, serum-free media and protein-free media. Actually, synthetic medium is an artificially designed medium (Yang & Xiong, 2012). Chemically Defined (CD) media does not contain proteins, hydrolysates or any other components of unknown composition. Growth factors or highly purified hormones either animal or plant origin can be added or can be supplemented as recombinant products (Brunner et al., 2010). In a chemically complex liquid medium, the cells are cultured which are suitable for supporting growth. For the development of specific cell type, there is numerous standard media formulation that has been developed (Sennott R.Stephen, 2004). Animal-Derived Component Free (ADCF) contains no component of animal origin. This type of medium is not necessarily chemically defined and it may contain bacterial or yeast hydrolysates or plant extracts. Serum-Free Media (SFM) contains discrete protein fractions like animal

tissue or plant extracts and is regarded as chemically undefined (Brunner et al., 2010). Lastly, the Protein-Free Media (PFM) where it does not contain high molecular weight proteins or protein fractions, but may contain peptide fractions (protein hydrolysates), and are thus not chemically defined. Protein-free media facilitates the downstream processing of recombinant proteins and the isolation of cellular products such as monoclonal antibodies (Kishishita et al., 2015).

2.8 Components of culture medium

2.8.1 Water

Water is the one of the crucial and critical components of a culture medium. Animal cells are very sensitive to water quality, because this can be the source of contamination that can affect cell growth. The water used for medium preparation has to be of high purity and should be prepared by efficient water purification systems such as multiple-distillation systems or equipment combining deionization, microfiltration, and reverse osmosis (M.Butler, 2004).

2.8.2 Carbohydrates

Glucose is the most common carbohydrate used in mammalian cell culture. Glucose is utilized as a part of most formulations to give an energy source and also a precursor for biosynthesis, for example, ribose required in nucleic acid synthesis. Glucose is present at an initial concentration of 10–25 mM. It reduces to all most half the level of concentration within a batch culture (Sennott R.Stephen, 2004).

2.8.3 Amino acids

As a source of precursors for protein synthesis, amino acids are incorporated at a concentration of 0.1–0.2 mM. In order to act as a precursor for the TCA cycle intermediates glutamine is generally included at higher concentrations (2–4 mM). But, ammonia is formed from the metabolic breakdown of glutamine and can be inhibitory to development in a few cultures (Sennott R.Stephen, 2004).

2.8.4 Vitamins and hormones

There are available at moderately low concentrations and are used as metabolic co-factors. Many vitamins especially B group vitamins are necessary for cell growth and proliferation and for some cell lines, the presence of B12 is essential. From one medium formulation to another the content of vitamins and hormones fluctuates enormously (Sennott R.Stephen, 2004).

2.8.5 Salts

The salts most commonly added to the culture medium sodium, magnesium, potassium, calcium, phospate, chloride, sulphate and bicarbonate These ions are important in the maintenance of the ionic balance and osmotic pressure, besides acting as enzymatic cofactors. Salts are the components that contribute most to the increase in culture medium osmolality (Freshney, 2005).

2.8.6 Buffering system

Most cells require pH conditions in the range 7.2-7.4 and close control of pH is essential for optimum culture conditions. There are major variations to this optimum. Most commercial culture media include phenol red as a pH indicator so that the pH status of the medium is constantly indicated by the colour (Freshney, 2005).

2.8.7 Antibiotics

For short-term cultures, antibiotics are regularly included in media because to minimize the risk of contamination. The ideal grouping concentration of antibiotics should be determined empirically because they might be cytotoxic. It is discouraged to use antibiotics for everyday subculture or in stock cultures. This is because low levels of bacterial or fungal contamination might be concealed and may bring problems later. Moreover, extensive use of antibiotics may bring about the selective retention of antibiotic resistant contaminants which can cause future problems (Sennott R.Stephen, 2004).

2.8.9 Glutamine

Many commercial culture media are readily supplemented with L-glutamine. Normally the concentration of L-glutamine used in standard media is 0.002mol/L (Yang & Xiong, 2012).

2.8.10 Serum

Serum is a natural medium obtained from animal body fluid extraction including plasma, lymph, and chicken embryos leaching solution. It contains rich nutrients, various somatomedin, hormones, osmotic pressure and pH of body environment (Yang & Xiong, 2012). Serum is normally used to provide mammalian cells with components necessary for survival. In most cases, the growth requirements of the cell are not met until serum is added to the culture medium at a concentration of five to ten percent. Serum can be obtained from various animal sources. One of the most effective components for cell growth is fetal calf serum because of its high content of embryonic growth factors (S.Ozturk, 2006).

Serum has a plethora of proteins, peptides, growth factors, hormones and other components which may be of significance for the cells to be cultivated and may not be present in the serum free media at all (Junaid Muneer Raja et al, 2011). The worldwide problem in the supply of serum are the pressure from biotechnology for simpler product filtration, and the requirement for reliable and characterized conditions for the culture of animal cell compelled the adoption of serum-free media (Liu & Chang, 2006).

Removal of serum from the cell culture medium is frequently carried out in order to remove various weakness posed by its usage which includes high physiological variability, high batch to batch variability, the risk of contamination and high cost, and challenges posed in the downstream processing of the product (Junaid Muneer Raja et al, 2011). Furthermore, serum-containing culture systems have become undesirable for large-scale processes in industry. Thus, in order to abolish the disadvantages the serum medium is gradually replaced by the synthetic medium (S.Ozturk, 2006).

The serum used in tissue culture is cattle serum. Cattle serum has several advantages includes adequate resource, mature preparation technique, and long

application time. Cattle serum includes bovine calf serum, newborn calf serum and Fetal Bovine Serum (FBS) (Yang & Xiong, 2012). The most broadly used serum is FBS with the combination of factors required for cell connection, growth, proliferation and it has been the supplement that effective for many types of human and animal cells. More than one million bovine foetuses must be harvested in order to sell roughly five hundred thousand litres of FBS per year. FBS manufacturing methods have come under inspection because of animal welfare concerns. Due to the animal welfare concern nowadays serum-free medium are mostly used in mammalian cell culture (Even et al., 2006).

2.9 Serum-free medium

Serum culture free media consist of a basal salt solution, such as a mixture of Ham's F-12 and Iscove's modified Dulbecco's Medium (DMEM) and other supplements such as carbohydrates, amino acids, vitamins, and minerals along with various growth supplements (Vetterlein, 1989). DMEM has a high nutrient concentration, non-essential amino acid and also trace elements such as selenium. These nutritional supplements are essential for improving metabolism, cell survival, and productivity, as well as protein expression levels (S.Ozturk, 2006).

In addition, the process of replacing serum in the medium has to meet the right combination of supplements to support the growth of the cells at a reasonable rate. Insulin transferrin, dexamethasone, Fibroblast Growth Factors (FGF), and albumin has been used for initial cultures of human endothelial cells are supplemented in serum-free medium (Sato, 1980).

The determination of a serum-free medium for a specific process of biopharmaceutical production using mammalian cells is a critical step for its success (Rodrigues et al., 2012). There is no single ideal serum-free medium formulation that works for all cell lines (Tan et al., 2015). Commercial serum-free and protein-free hybridoma medium used as a basal medium is supplemented with phosphatidylcholine, cholesterol, â-cyclodextrin, and ferric citrate (Spens & Ha, 2005).

A recent review of animal cell cultures identified serum-free media as a vital development that needs to be addressed to ensure the future success of animal cell

culture processes (Even et al., 2006). Amino acids, vitamins, trace metals, sugars, salts, buffers, growth factors, and various other components naturally consist in the cell culture media and have shown to affect cell growth, antibody titer, and product quality (Kishishita et al., 2015). The serum-free medium also must have enzyme inhibitor to stop the activity of trypsin and thus protect the cell (Yang & Xiong, 2012).For the advancement of serum free medium, biotechnologists normally weaned animal cells from the serum supplemented. Consecutively, they have to simultaneously screen the components and find their optimal concentrations that will support the growth of the cells under the serum-free condition. This study used pre-weaning approach to increase the probability of thriving growth in the development process for serum-free medium (Liu & Chang, 2006).

There are numerous examples of the application of serum-free media to primary culture (Sato, 1980).. Medium developed for Sertoli cells will allow survival from primary cultures of immature mouse testes with no fibroblastic over growth. There are few commercially available serum-free media such as EX-CELL, ISF-I, CD CHO, CDM4CHO, CHO-III-A, Octomed and HybridoMed were evaluated and compared for cell growth and monoclonal antibody (mAb) production of a transfected CHO-K1 cell line (Liu & Chang, 2006).

Adaptation of cells to serum-free could be accomplished in stationary T-flasks throughout the sequential adaptation process. The gradual serum reduction increases the chances of acceptable cell adaptation to serum-free culture (Ma & Tage, 2015). In the sequential adaptation, cells are transferred into a mixture of serum-containing and serum-free medium, until the complete serum free condition is achieved. It is suggested to keep the cell culture in a ten-percent serum-containing and ninety-percent serum-free medium mixture for 2–3 passages, before changing to a complete serum-free medium (Kishishita et al., 2015).

CHAPTER 3

METHODOLOGY

3.1 Introduction

In this chapter, the experimental materials and methods that were used in the adaptation process are reported in detail.The chemicals and methods used in this study were adapted from various journals, articles and literature studies.

3.2 Material

3.2.1 Chemicals and reagents

The following chemicals and reagents were used: Dulbecco's Modified Eagle's Medium (DMEM) powder (Life Technologies, USA), Fetal Bovine Serum (Life Technlogies, South America), 1% (v/v) penicillin (Life Technologies, USA), Trypsin ((Life Technologies, Canada), PBS (Life Technologies, USA), Trypan Blue solution (Life Technologies, USA), serum- free media, EX-CELL Chemically Defined (Sigma Aldrich, USA), Hydrochloric acid (R&M chemical, UK) and Sodium Hydroxide (Fisher Scientific, UK),Sodium Bicarbonate(Sigma Aldrich, USA), Trypan blue(Sigma Aldrich, Germany), Ultra purified water (Elix Technology, French),ethanol (R&M chemical, UK), and Decon 90 (Fisher Scientific, England).

3.2.2 Laboratory equipment

The following equipment was used during project: Biosafety cabinet (Esco, USA), CO_2 incubator (New Brunswick, UK), Neubauer counting chamber(Assistant, Germany), Refrigerated centrifuge (Eppendorf AG, Germany), centrifuge tube (Thermoline, Germany), ultra-purified water system (Elix Technology, French), inverted phase microscope (Life technologies, USA), magnetic stirrer (Thermo Scientific, China), pH meter (Mettler Toledo, China), microbalance (Mettler Toledo, China), 96-well plate (Thermo Scientific, China), T-flask (Thermo Scientific, Denmark), vacuum pump (Fisherbrand, USA), pipette (Eppendorf, Germany), chiller (Thermo Scientific, USA), autoclave (Alp, Japan), and Biochemical analyser (YSI, USA).

3.2.3 Cell type

Anti-CEAhybridoma cell line was obtained from InnoBiologics Sdn. Bhd. These cells secrete humanized anti-carcino embryogenic antigen (CEA).

3.3 Overview of Experimental Study

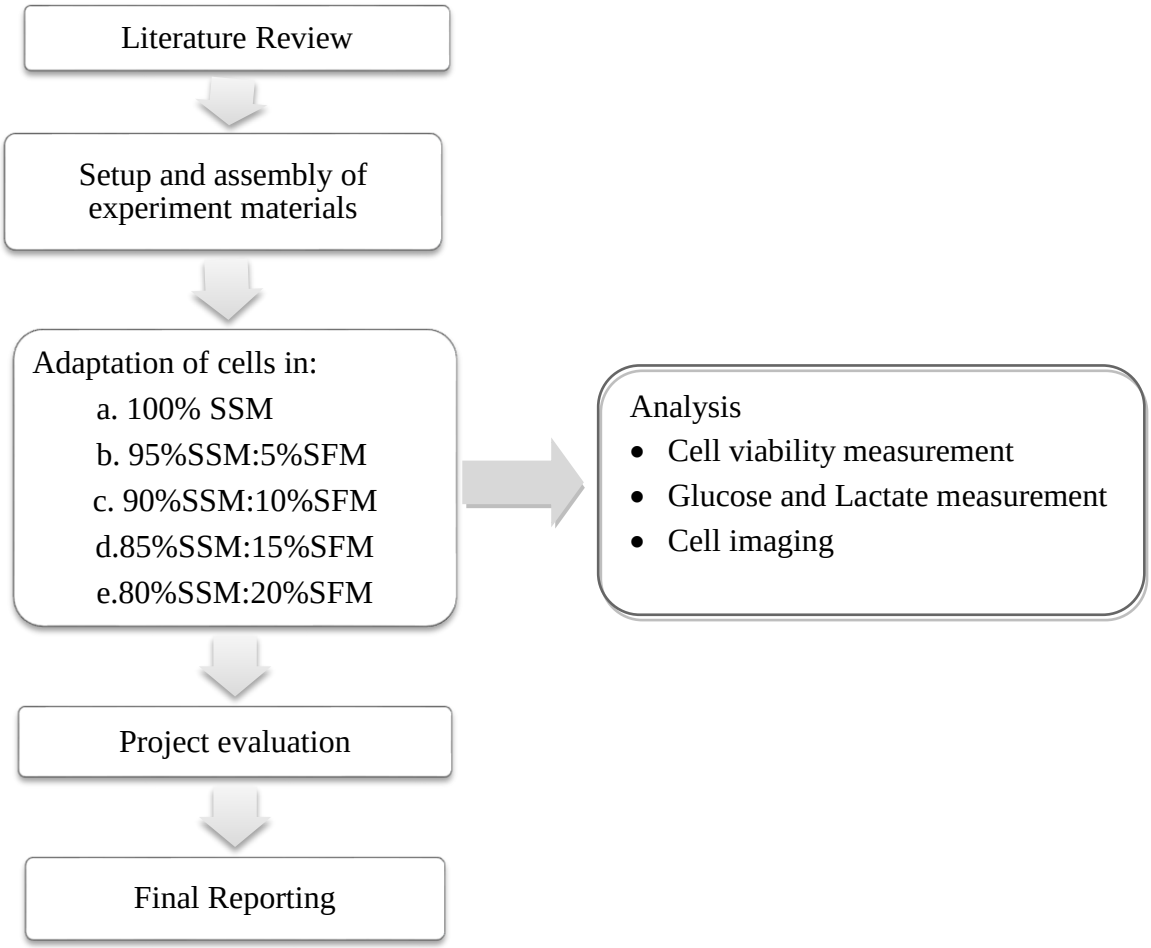


Figure 2: The overview of experimental study

3.4 Methods

3.4.1 Preparation of media from powder

First, powdered medium were added to tissue culture grade. The mixture was gently stirred until all powdered medium are dissolved. Then, the required amount of buffer and other additives were added. The pH was adjusted to pH 7.2 using 1M sodium hydroxide (NaOH) or 1M hydrochloric acid (HCl) and it was stirred gently. The mixture was aseptically filtered and the prepared medium was later stored at 4˚C chiller.

3.4.2 Cell Line and Routine Culture Conditions

The cell line was maintained in cryo vials until usage. The cells were routinely grown in static culture using 25 cm2 T-flasks containing 5 ml of DMEM medium supplemented with 10% (v/v) serum, 2 mM L-glutamine, and 1% (v/v) penicillin. Cells were incubated at 37oC under humidified 5% CO2 atmosphere. The cells were cultured in mixtures of different ratios of Serum Supplemented Medium (SSM) to Serum-Free Medium (100:0; 95:5; 90:10; 85:15 and 80:20). When the cells reach a density of about 1 x 106cells/ml or more trypsinisation mehod need to be done using trysin. Trypsin is a proteolytic enzyme which breaks down the protein that attached to the culture surfacewhere it is added to the flask containing cells for a while in order to wash the media. For each adaptation, cells were seeded at seeding density of 1 x 105cells/ml (Junaid Muneer Raja et al, 2011).

3.4.3 Cell revival from cryopreservation

The cryovial containing the frozen cells were removed from liquid nitrogen storage. The cells were quickly thawed by gently swirling the vial until there is just a small bit of ice left in the vial. The vial was later wiped with 70% ethanol before transferred into the bio safety cabinet. Next, the thawed cells were transferred drop wise into the centrifuge tube containing the desired amount of pre-warmed complete growth medium appropriate for the cell line. The cell suspension was centrifuged at approximately 200×g for 5 minutes. After the centrifugation, the clarity of the supernatant and visibility of a complete pellet was checked. The supernatant was aseptically removed without disturbing the cell pellet. Cell pellet were gently re-suspended in complete growth medium, and then transferred into an appropriate culture vessel before incubated at 37˚C (Invitrogen, 2010).

3.4.4 Subculture

The cultures were viewed by an inverted phase microscope to observe the degree of confluence and to check the absence of bacterial and fungal contaminants. The spent medium was removed and the cell monolayer was washed with PBS. 1ml of trypsin was later pipetted onto the washed cell monolayer. The flask was swirled to ensure the trypsin completely covered the cell monolayer. The flask was incubated for 3-5 minutes. The cells were examined using a microscope to make sure all cells have been detached. The side of the flasks was gently tapped to release the remaining attached cells. Few amounts of cultures were later removed to perform cell count. Lastly, cells at required concentration were transferred to a newly labelled flask containing the growth medium. The cultures were incubated and the process was repeated as demanded by the growth characteristics of the cell line (Aldrich, 2010).

3.4.5 Adaptation of cells to serum-free medium by slow adaptation

Cells in the medium with varying concentrations of serum with ratio of 95%SSM :15%SFM, 90%SSM:10%SFM, 85%SSM:15%SFM and 100% SFM were cultured by slow adaptation method as shown in figure 3.

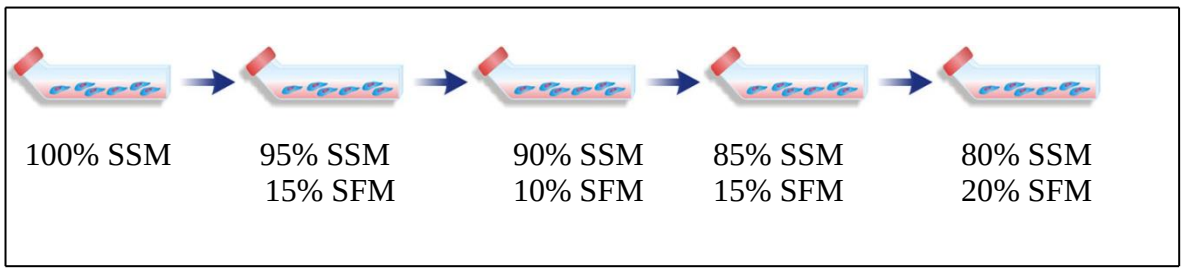


Figure 3 : Schematic representation of the serum-free media adaptation process in static T-flasks (figure adapted from Biaggio et. al, 2015)

The cells grown were sub-cultured in the 5ml of Serum Supplemented Medium by reducing the concentration of Serum Supplemented Medium from 100% SSM until the 80% SSM. When the cell viability reach 1 to 3 x 10^6viable cells per ml, the cells were sub-cultured again in different concentration of SSM with an inoculation size of 1-2x10^5viable cells per ml

with at least 85-percent viability. The cells were considered as fully adapted when the viable cell count exceeds 4 x 10^6 viable cells per ml after 3 days.

3.5 Analytical testing

3.5.1 Cell counting using trypan blue exclusion method

The mixture of the cell suspension and 0.4% (v/v) trypan blue solution were made. The mixture was gently mixed and was let stood for 5 min at room temperature. Both hemocytometer and coverslip were washed with 70% (v/v) ethanol and were allowed to dry. The coverslip was placed on top of the hemocytometer counting chamber. 10 millilitres of cell suspension was applied to the edge of the chamber between the cover slip and the V-shaped groove on the chamber. The cell suspension was drawn into the chamber by capillary action and the number of cells was counted under appropriate magnification(Walker, 2011).

Cell counting using haemocytometer

The number of cells for both viable (unstained) and nonviable (stained) was counted in each of the four corner quadrants (A, B, C, D). The average of these four readings was taken and multiplies by 10^4 to obtain the number of cells per mL in the sample applied to the haemocytometer. Next, it was multiply by two to take into account the 1:1 dilution of the sample in the trypan blue. Again it was multiply by any dilutions in the original sample preparation of the cell suspension.

$$\text{Number of cells} = \frac{(\text{A} + \text{B} + \text{C} + \text{D})}{4} \text{X} 10^4 \text{X 2 X sample dilution}$$

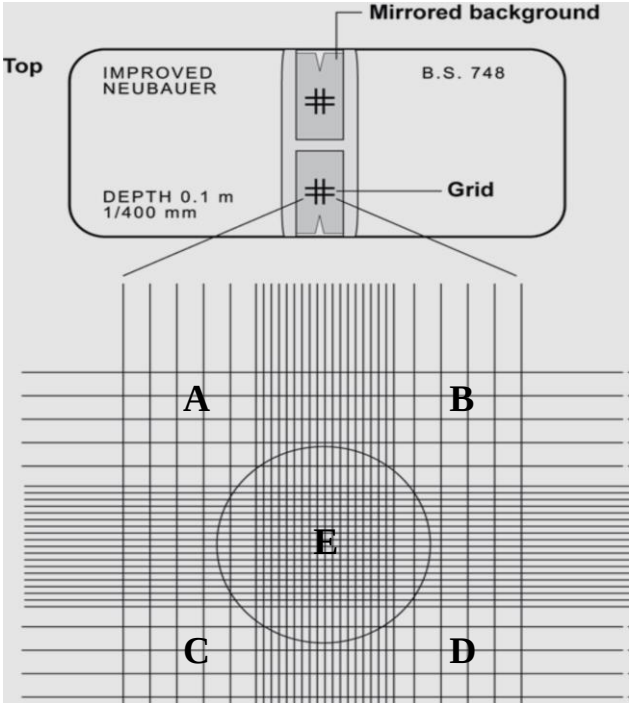


Figure 4: representation hemacytometer slide. The four corner squares (A, B, C, and D) and the central square (E) are counted on each side of the hemacytometer (figure adapted from Invitrogen, 2010)

The percentage of unstained cells represents the percentage of viable cells in the suspension (Walker, 2011).

$$\text{Percentage of Viable cells} = \frac{\text{number of viable cells}}{\text{total number of cells}}$$

3.5.2 Determination of the glucose and lactate concentration

The concentration of glucose and lactate were determined using spent medium that were collected during cell counts. Using 96 well plate, 125µl of samples were analyse using bio chemical analyzer. The data obtained was used to metabolic profiling.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

The aim of presenting this chapter is to have the obtained research tabulated based on the methods presented in Chapter 3. In this chapter,all results including the cell density, cell viability, metabolic profiles and cell morphology were continuously discussed. The results were presented in various formats such as tables and graphs.

4.2 Cell culture in 100% SSM

Figure 3.1 shows the growth profile of anti-CEA hybridoma cells in 100% SSM. All T-flask were inoculated at a seeding density of 1.0×10^5cells/mL. The cells had entered the exponential phase directly after the cell inoculation which is from day 1 until day 5. Within those days, the cells multiply rapidly, and cell mass and cell number density increase exponentially with time. On day 5 the cell reached high cell density and after day 5 deceleration phase occurs in the medium. This is due to depletion of one or more essential nutrients or the accumulation of toxic by-products of growth. The cells reached a peak cell density of 1.3×10^6 cells/mL with a cell viability of 92.2 % on day 5. On the 8^{th} day the cells density reduced to 7.1×10^5 cells/mL with cell viability of 47%. The glucose was almost completely utilised from day 1 until day 8 during the growth of cells. When the cells reached its maximum cell density on day 5 the glucose concentration dropped with a concentration of 2.2 g/L. The amount of lactate produced on day 5 was 0.71 g/L and on day 8 the lactate produced was 0.91 g/L.

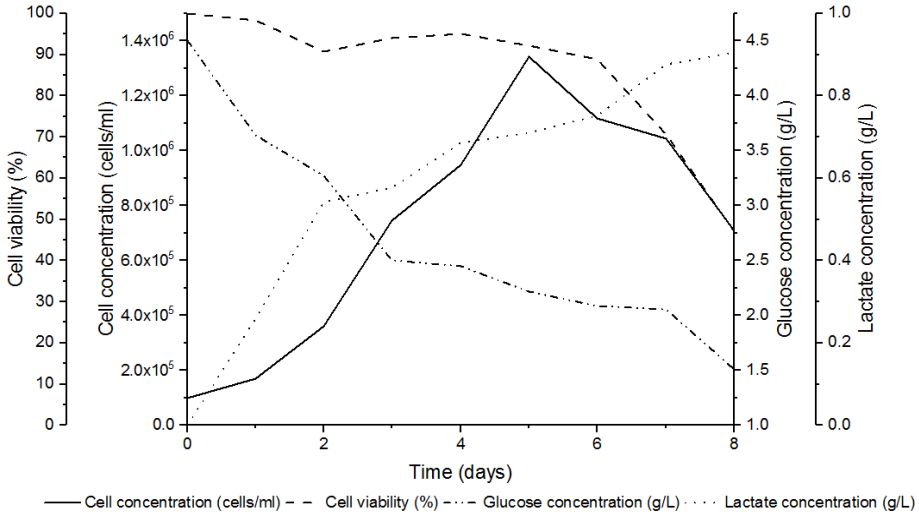


Figure 3.1: Graph of cell viability, cell concentration, glucose, and lactate concentration against time in 100% SSM.

Overall the anti-CEA hybridomas cells were spherical in shape with unequal sizes, and have a bit granular appearance. On day 1 the cells were just started to adapt in the culture medium as evedenced by the image in which only few cells can be seen. On day 2 , day 3 and day 4 the density of cells started to increase and the space between the cells and the medium became less. On day 5, the culture appeared to have become fully confluent. Then, on day 6 and day 7 the size of the cells was shrinking as there was no space for the cells to growth further. On the last day which is on day 8, almost all the cells were died and the shapes of the cells still observed as rounded.

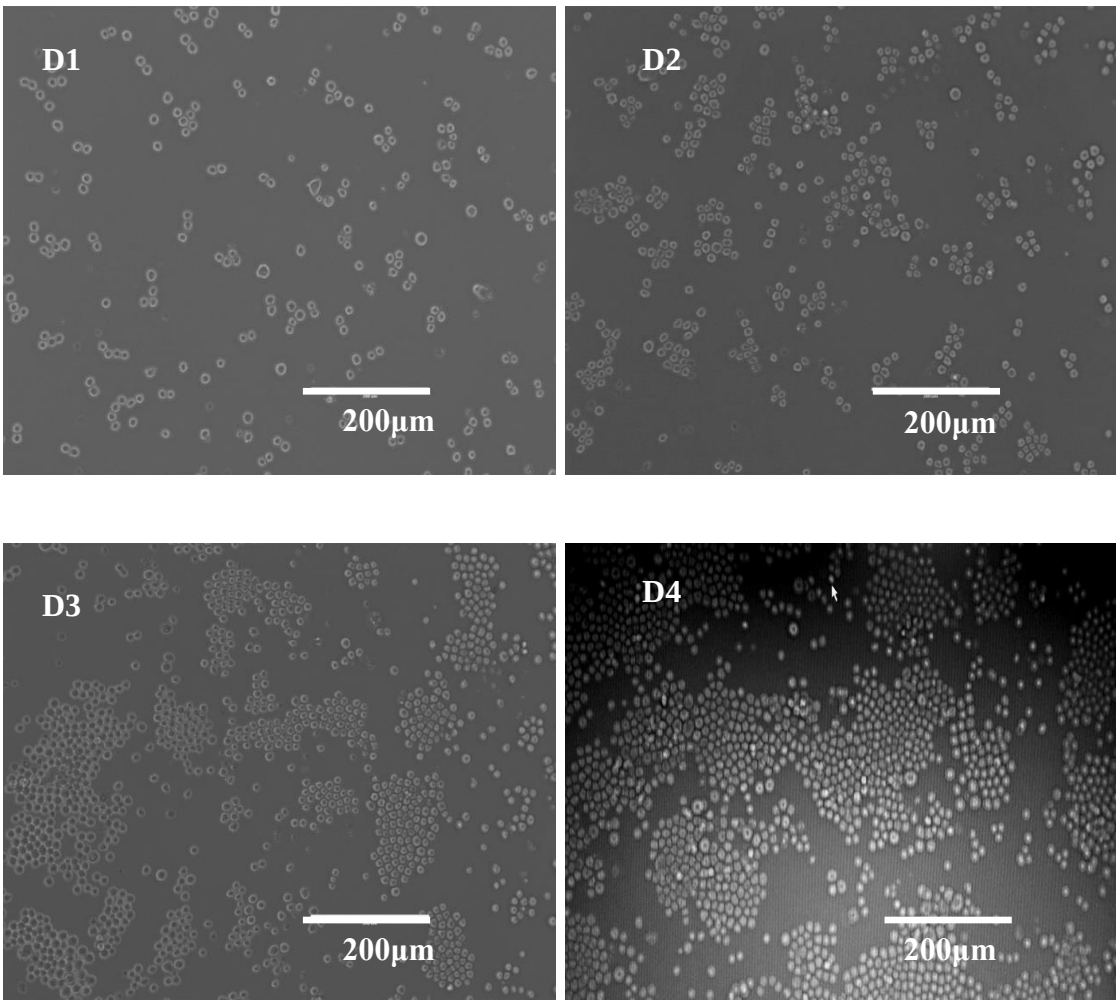


Figure 3.1 a: Microscopic observation of the cells morphology in 100% SSM on day 1, day 2, day 3 and day 4

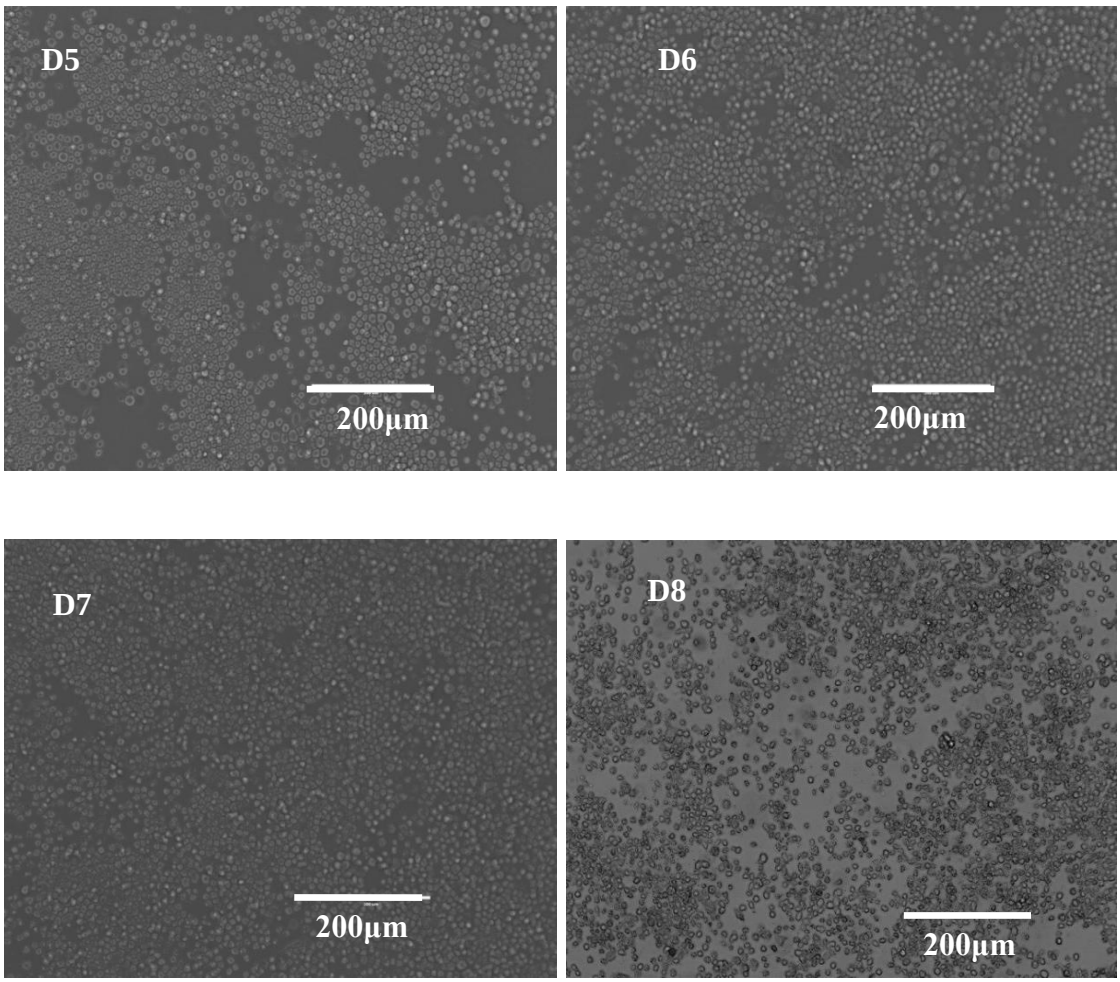


Figure 3.1 b: Microscopic observation of the cells morphology in 100% SSM on day 5, day 6, day 7 and day 8

4.3 Adaptation of cells in 95% SSM: 5% SFM

Figure 3.2 shows the growth profile of anti-CEA hybridoma cells in 95% SSM: 5% SFM**.** The cells had a lag phase from day 0 until day 3. The cells had entered the exponential phase directly after the cell inoculation after day 3. On day 5 the cell reached high cell density and after day 5 deceleration phase occurs in the medium. . The cells reached a peak cell density of 8.88×10^5cells/mL with a cell viability of 84.7 % on day 5. On the 7th day the cells density reduced to 3.13×10^5cells/mL with cell viability of 85.6%. It is observed that, on the 8th day the cells enter death phase where almost all the cells were died and the cell viability obtained was zero. This is because of either nutrient depletion or toxic product accumulation in the medium. The glucose was almost completely utilised from day 1 until day 8 during the growth of cells. When the cells reached its maximum cell density on day 5 the glucose concentration dropped with a concentration of 2.36 g/L. The amount of lactate produced on day 5 was 0.42g/L and on day 8 the lactate produced was 0.90 g/L.

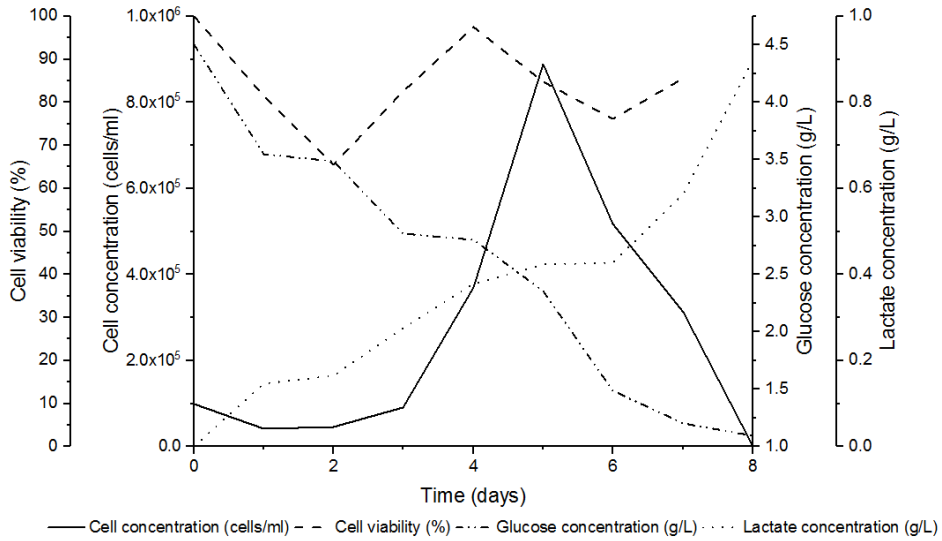


Figure 3.2: Graph of cell viability, cell concentration, glucose, and lactate concentration against time in 95% SSM: 5% SFM.

On day 1 the cells were just started to adapt in the culture medium as evedenced by the image in which only few cells can be seen. On day 2 , day 3 and day 4 the density of cells started to increase but not really covered the suface of T- flask. On day 5, the culture appeared to have become fully confluent and the space between the cells and the medium became more compact. Then, on day 6 and day 7 the size of the cells was shrinking as there was no space for the cells to growth further. On the last day which is on day 8, almost all the cells were died and the shapes of the cells still observed as less rounded.

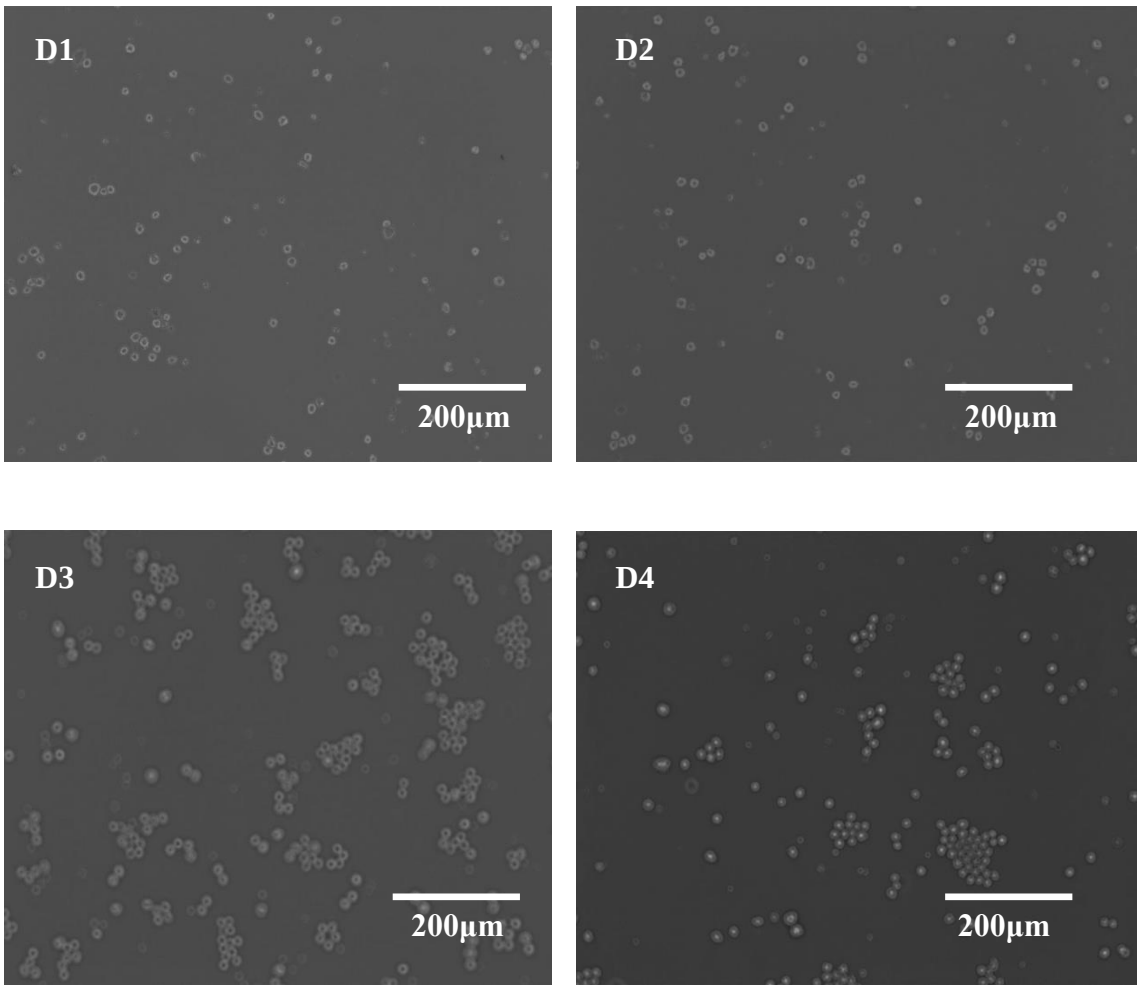


Figure 3.2 a: Microscopic observation of the cells morphology in 95% SSM: 5% SFM on day 1, day 2, day 2 and day 4.

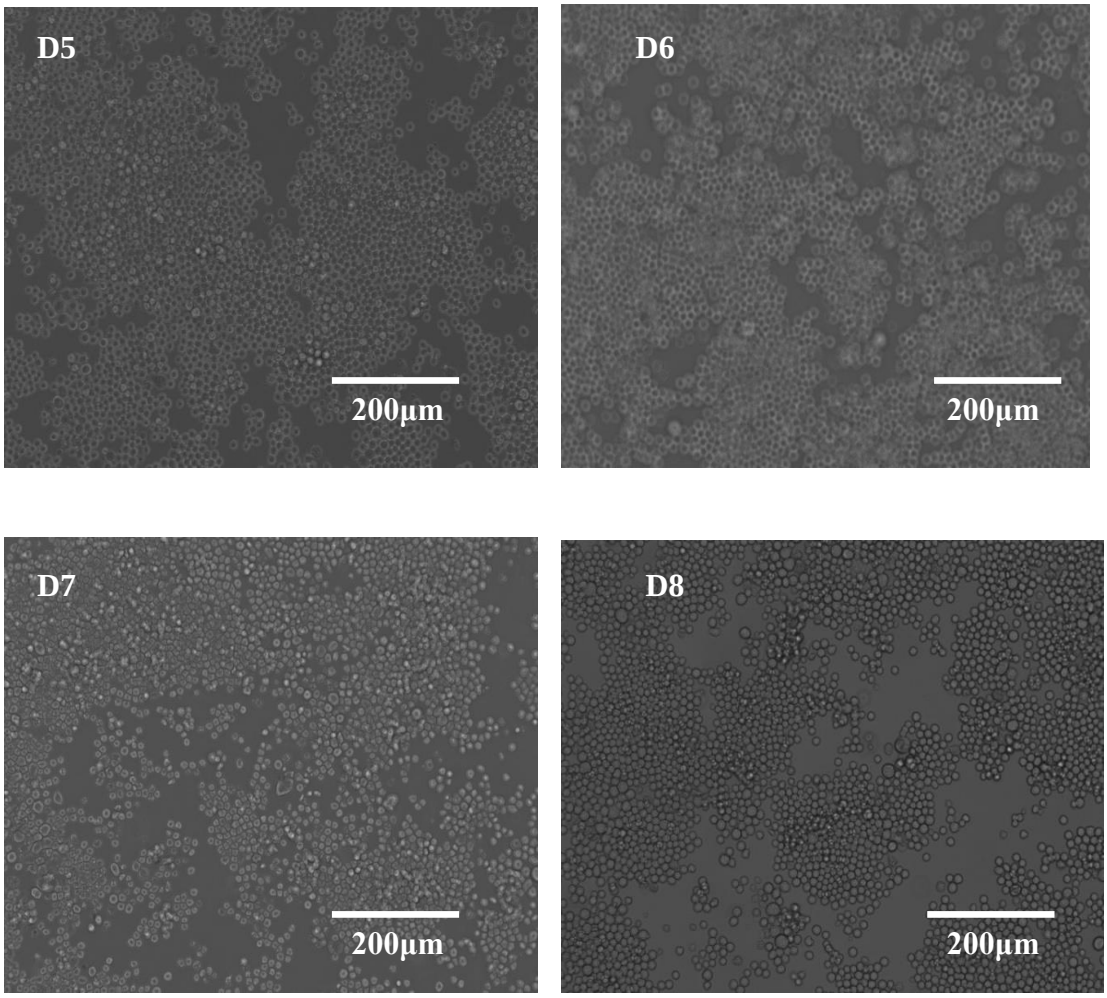


Figure 3.2 b: Microscopic observation of the cells morphology in 95% SSM: 5% SFM on day 5, day 6, day 7 and day 8.

4.4 Adaptation of cells in 90% SSM: 10% SFM

Figure 3.3 shows the growth profile of anti-CEA hybridoma cells in 90% SSM: 10% SFM**.** The cells had a lag phase for 24 hours. There is no clear increment in cell concentration during the period. Then, the cells had an exponential growth until day 5 and by day 8 most of the cells were dead in this medium. The adaptation of cells started at a seeding density of 1.0×10^{5}cells/mL. Cells reached a peak cell density of 1.07×10^{6}cells/mL with a cell viability of 87.8 % on day 5. On the 7th day the cells density reduced to 5.6×10^{5}cells/mL with cell viability of 80.7%. It is observed that, on the 8th day almost all the cells were died and the cell viability obtained was zero. The glucose was almost completely utilised from day 1 until day 8 during the growth of cells. When the cells reached its maximum cell density on day 5 the glucose concentration dropped with a concentration of 3.0 g/L. The amount of lactate produced on day 5 which is 0.19g/L and on day 8 the lactate produced was 0.22 g/L.

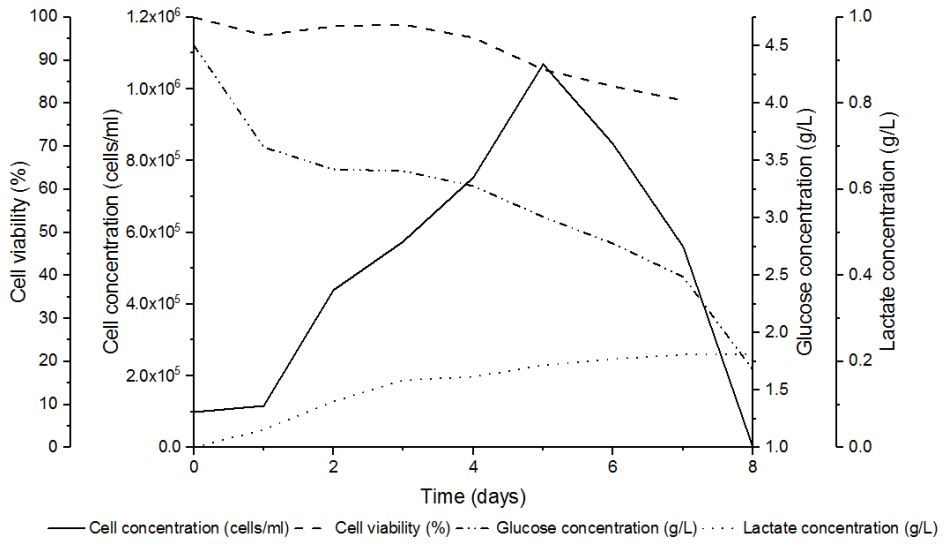


Figure 3.3: Graph of cell viability, cell concentration, glucose, and lactate concentration against time in 90% SSM: 10% SFM.

On day 1 the cells just start to adapted where from the image we can observed that only few living cells can be identify and have more space in the medium and same goes to the cells on day 2 and day 3. But on day 4 only the density of cells started to increase, much healthier cells were observed and the space between the cells and the medium became less. On day 5, cells appeared to be confluent and reached its maximum cell density where many living cells were observed. Then, on day 6 and day 7 the size of the cells was shrinking and there is no space for the cells to growth further. On the last day which is on day 8, almost all the cells were died, the shapes of the cells were irregular and only few the cells were observed.

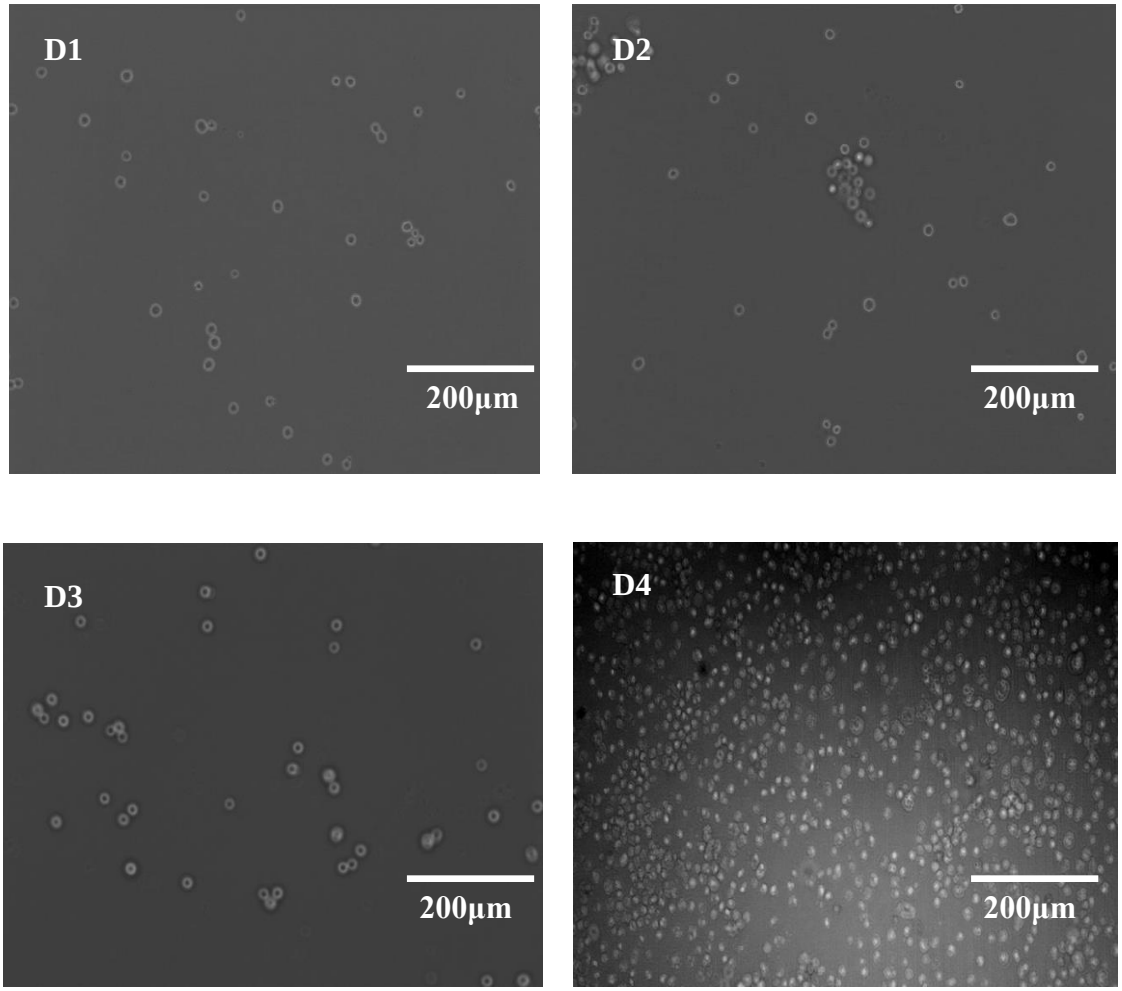


Figure 3.3 a: Microscopic observation of the cells morphology in 90% SSM: 10% SFM on day 1, day 2, day 3 and day 4

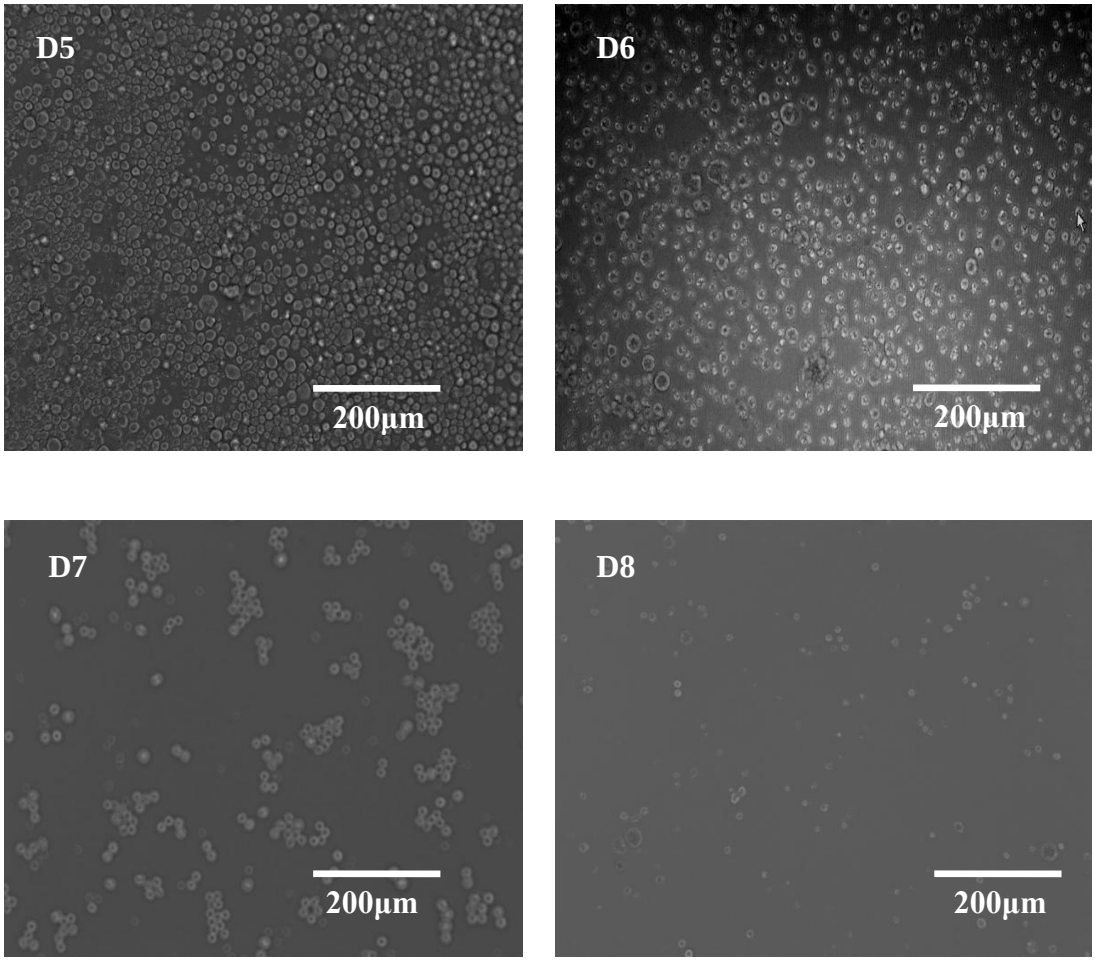


Figure 3.3 b: Microscopic observation of the cells morphology in 90% SSM: 10% SFM on day 5, day 6, day 7 and day 8

4.5 Adaptation of cells in 85% SSM: 15% SFM

Figure 3.4 shows the growth profile of anti-CEA hybridoma cells in 85% SSM: 15% SFM**.** The cells had a lag phase for 24 hours. There is no clear increment in cell concentration during the period. Then, the cells had an exponential growth until day 5 and by day 8 most of the cells were dead in this medium. Cells reached a peak cell density of 8.62×10^{5}cells/mL with a cell viability of 92.8 % on day 5. On the 7^{th} day the cells density reduced to 2.45×10^{5}cells/mL with cell viability of 67.1 %. It is observed that, on the 8^{th} day almost all the cells were died and the cell viability obtained was zero. The glucose was almost completely utilised from day 1 until day 8 during the growth of cells. When the cells reached its maximum cell density on day 5 the glucose concentration dropped with a concentration of 3.5 g/L. The amount of lactate produced on day 5 which is 0.25g/L and on day 8 the lactate produced was 0.45g/L.

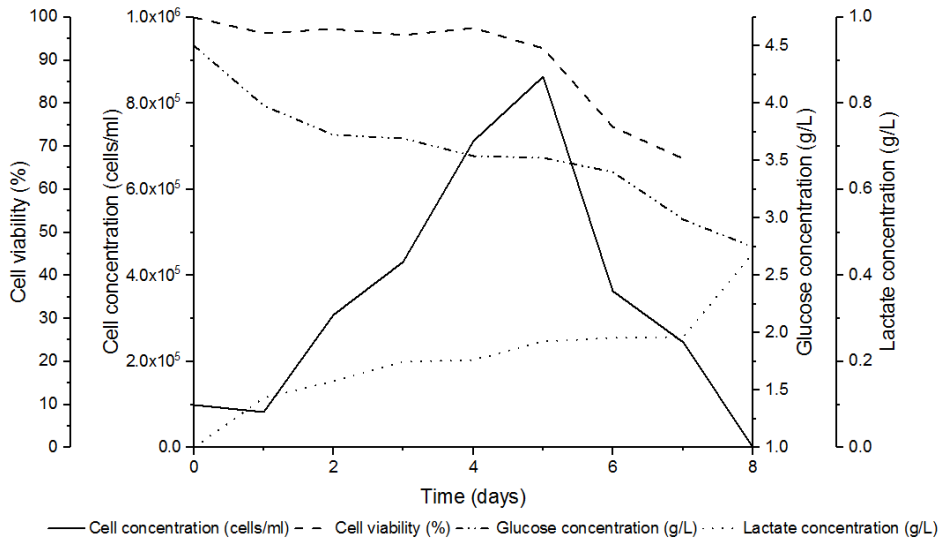


Figure 3.4: Graph of cell viability, cell concentration, glucose, and lactate concentration against time in 85% SSM: 15% SFM.

On day 1 the cells just start to adapted where from the image we can observed that only few living cells can be identify and have more space in the medium. On day 2 and day 3 the density of cells started to increase and the space between the cells and the medium became less. On day 4 the cells were formed in clumps and started to replicates.The cell density of was lesser than in 90% SSM. On day 5, cells appeared to be confluent and reached its maximum cell density where many living cells were observed. Then, on day 6 and day 7 the size of the cells was shrinking and there is no space for the cells to growth further. On the last day which is on day 8, almost all the cells were died, the shapes of the cells were irregular.

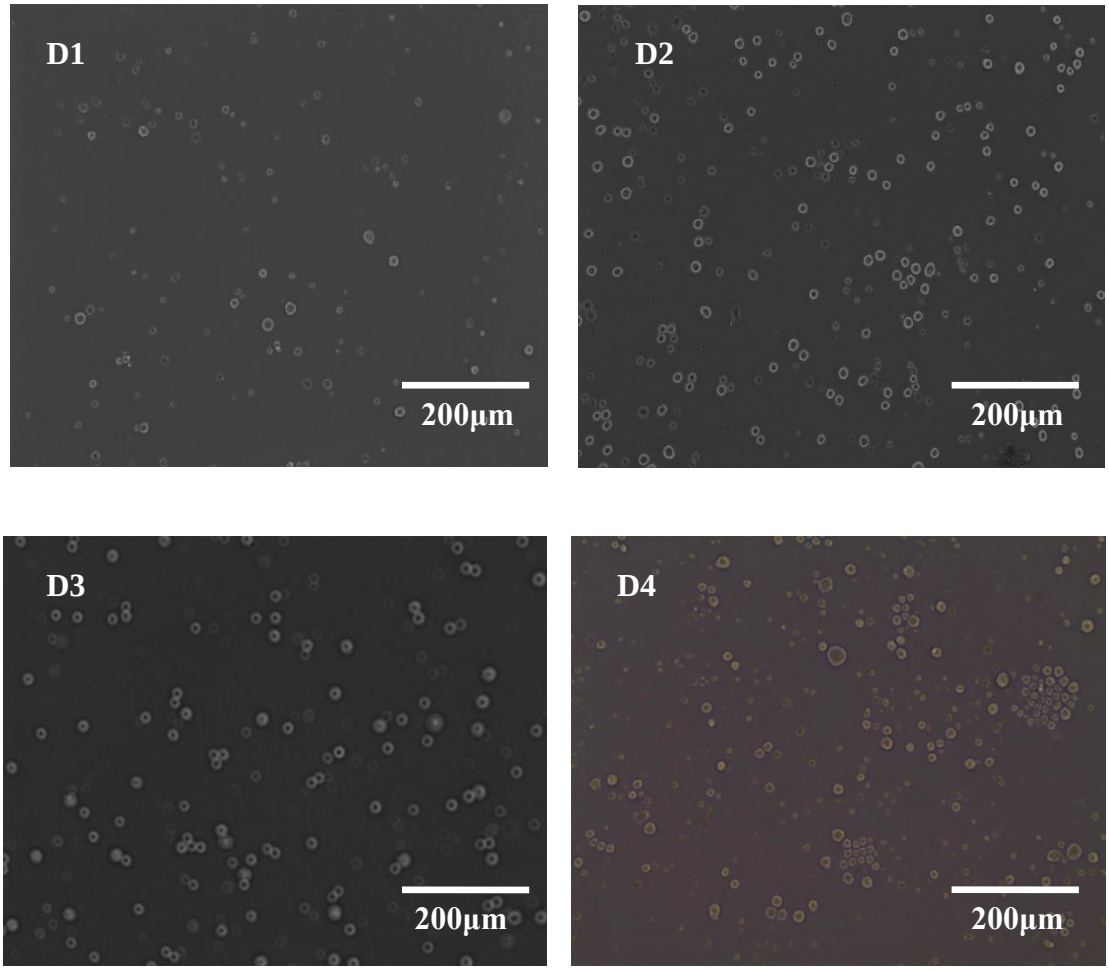


Figure 3.4 a: Microscopic observation of the cells morphology in 85% SSM: 15% SFM on day 1, day 2, day 3 and day 4

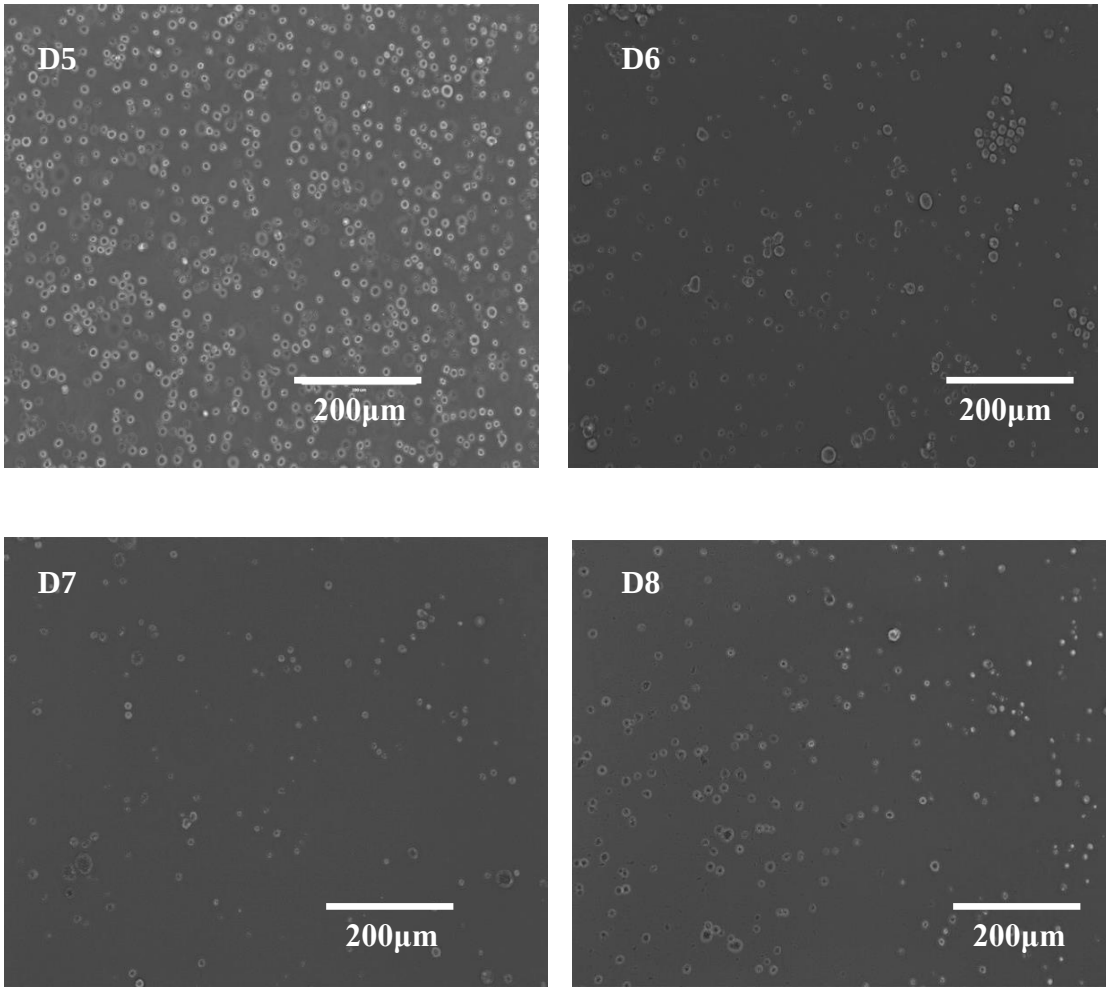


Figure 3.4 b: Microscopic observation of the cells morphology in 85% SSM: 15% SFM on day 5, day6, day 7 and day 8

4.6 Adaptation of cells in 80% SSM: 20% SFM

Figure 3.5 shows the growth profile of anti-CEA hybridoma cells in 80% SSM: 20% SFM**.** The cells had a lag phase for 24 hours. Then, the cells had an exponential growth until day 4 and by day 8 most of the cells were dead in this medium. The adaptation of cells started at a seeding density of 1.0×10^5cells/mL. Cells reached a peak cell density of 7.55×10^5cells/mL with a cell viability of 95.0 % on day 4. On the 7th day the cells density reduced to 1.47×10^5cells/mL with cell viability of 63.7 %. It is observed that, on the 8th day almost all the cells were died and the cell viability obtained was zero. When the cells reached its maximum cell density on day 4 the glucose concentration dropped with a concentration of 3.5 g/L. The amount of lactate produced on day 4 which is 0.26g/L and on day 8 the lactate produced was 0.28g/L.

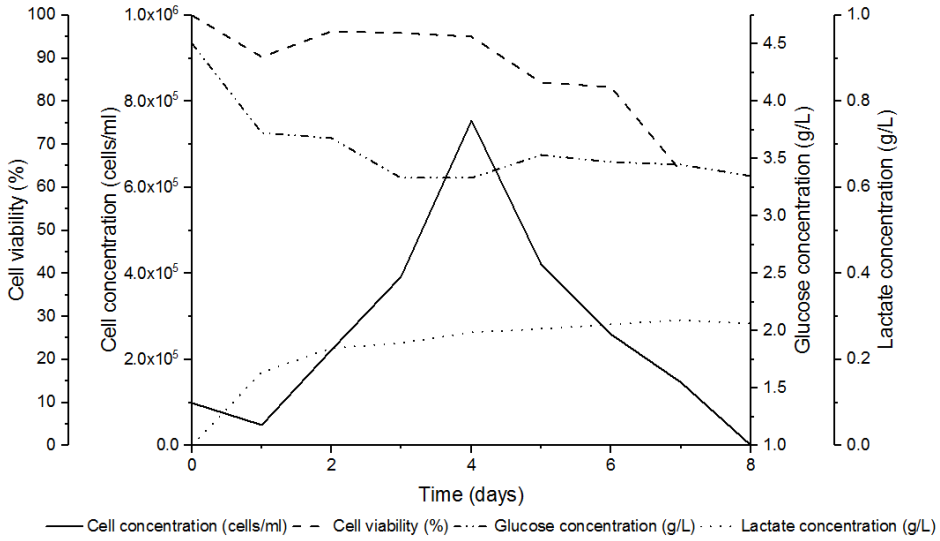


Figure 3.5: Graph of cell viability, cell concentration, glucose, and lactate concentration against time in 80% SSM: 20% SFM.

On day 1 the cells just start to adapted where from the image we can observed that only few living cells can be observed and have more space in the medium. On day 2 , day 3 and day 4 the density of cells started to increase. There is a space for the cells to grow but due to the cells are cannot adapt in the 80% SSM, the cells were not so conflunt as in previous conditions. So, the cell density of the cells were lesser than in 85% SSM. On day 5, cells appeared to be confluent and reached its maximum cell density where many living cells were observed. Then, on day 6 and day 7 the size of the cells was shrinking although there is an enough space for the cells to grow, the cells started to die. On the last day which is on day 8, almost all the cells were died, the shapes of the cells were irregular and the cells were not healthy.

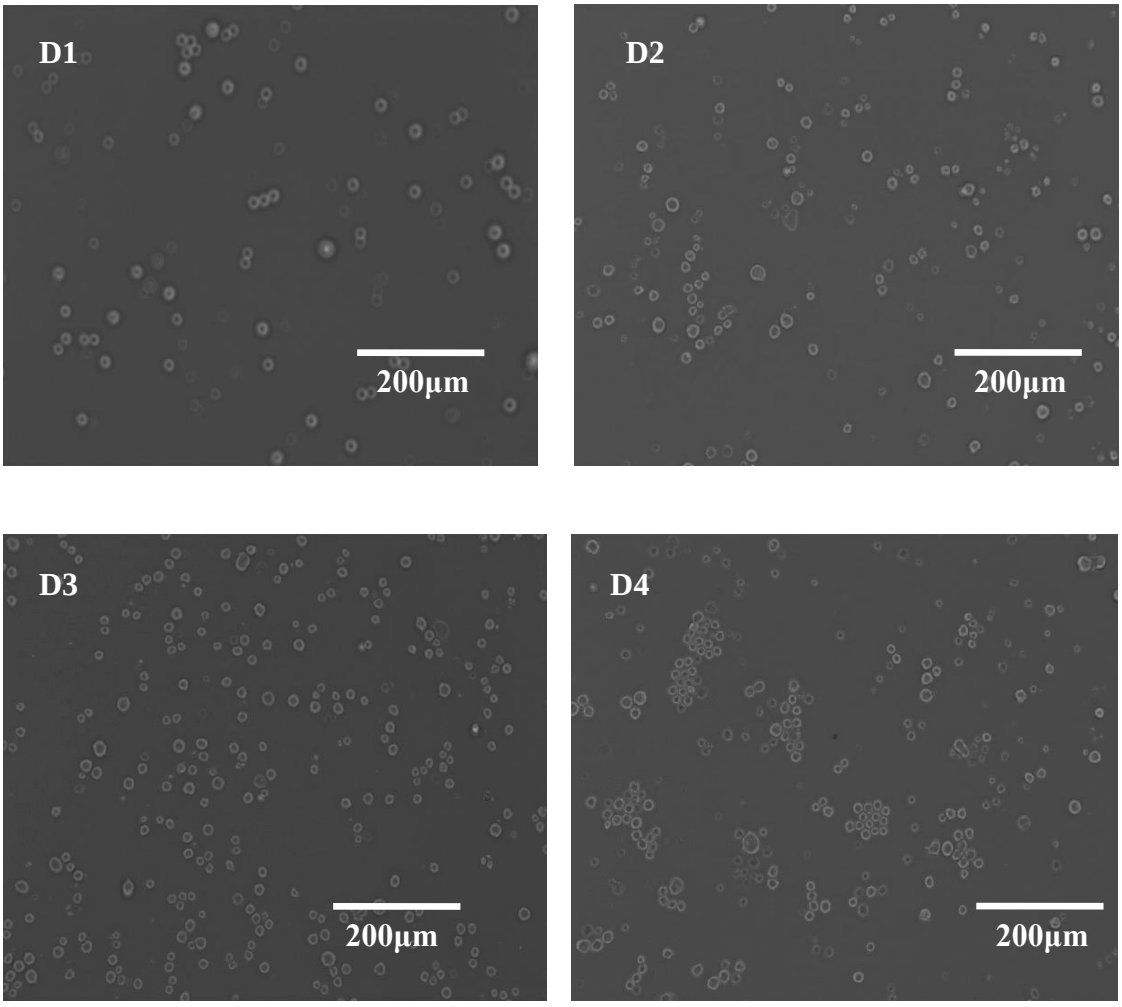


Figure 3.5 a: Microscopic observation of the cells morphology in 80% SSM: 20% SFM on day 1, day 2, day 3 and day4

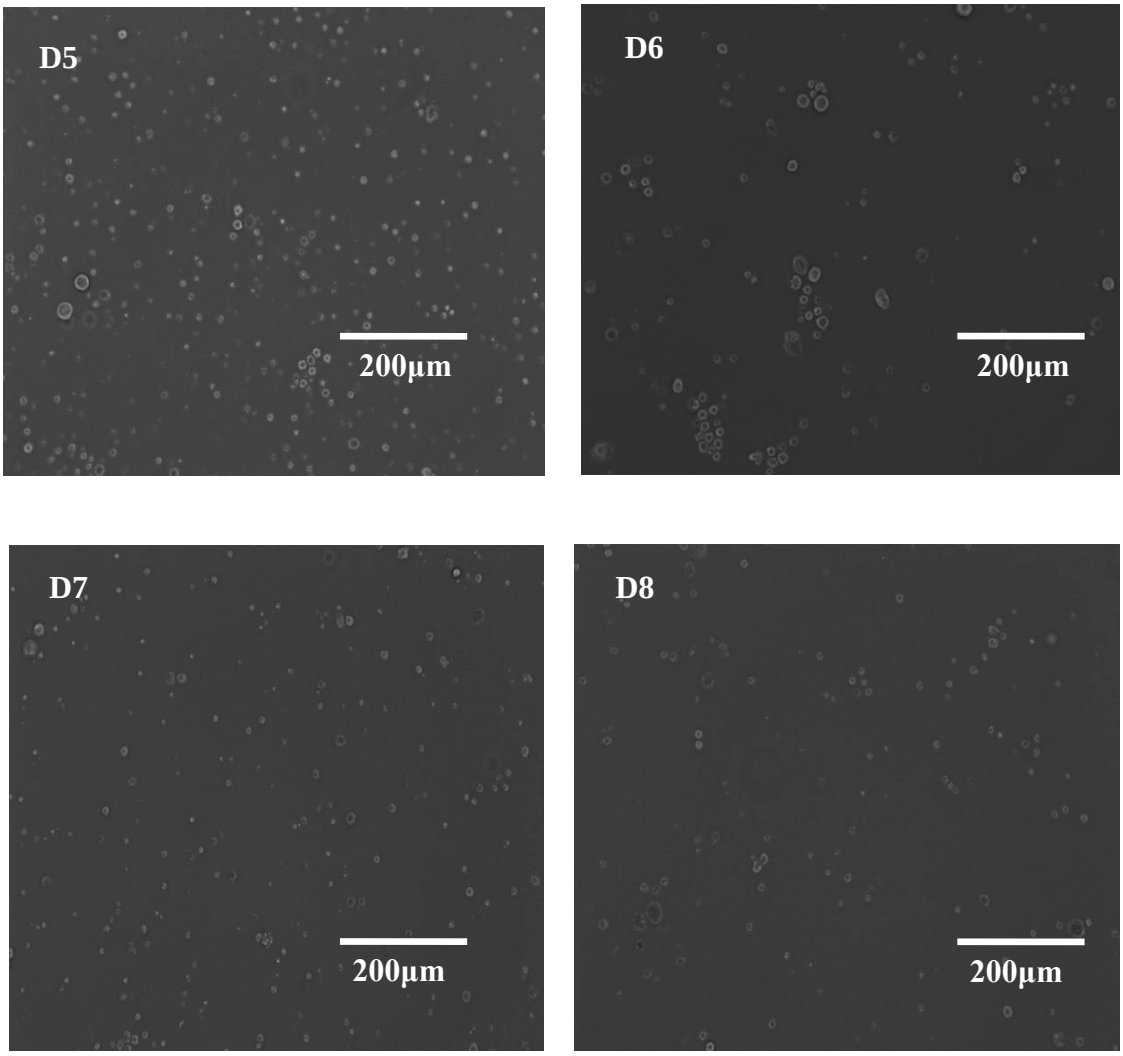


Figure 3.5 b: Microscopic observation of the cells morphology in 80% SSM: 20% SFM on day 5, day 6, day 7 and day 8

4.7 Comparison of cell growth profiles in all adaptations

Table 1 shows the peak cell density achieved in all medium compositions. All the conditions except in 100% SSM, exhibited a slower proliferation rate after adapted to low serum contain medium. At 80% SSM the cell density reached the maximum cell density on day 4 but for other conditions the maximum cell density was reached on day 5.

It is found that the 100 % SSM would maintain acceptable cell growth and differentiation. So, when comparing with 100% SSM with other ratios, the trend of growth profile of cells was similar but the values of cell density for each adaptation ratio is getting smaller. It indicates that at the lower percentage of serum the cells cannot grow healthily and the density of the cells is much lesser. This is because serum prevents cell death through promoting cell proliferation and survival by either acting as shear protecting agent or protection of cells against apoptosis based on its rich nutrient composition. Another reason for this could be the absence of serum which is the FBS where FBS is a mixture rich of growth factors, proteins, vitamins, trace elements, hormones, etc. Serum proteins serve as a carrier for lipids, enzymes, micronutrients, and trace elements into the cell (Broedel, S. E., & Papciak, S. M.2003).

As an evident from the table 1 below, in 100% SSM, the cell density reached is1.34×10^{6} cells/ml on day 5.But when we comparing it with 80% SSM, the cells do not grow well and the peak cell density reached is 7.55×10^{5}cells/ml which is the lowest at day 4. But in 95% SSM the cell density was slightly lower than in 90% SSM. The reason for that problem might be because the cell lines originally growing adherently might lose its ability after adapted in different percentage of serum (95% SSM) and begin to grow in different percentage of serum (90% SSM) (Hernández & Fischer, 2006). After the adaptation in 90% SSM onwards the cells were stabilized but the growth rate were decreased as percentage of serum reduce.

Overall it can be observed that the cell growth profiles for all composition of culture medium displayed similar trend. Nevertheless, the peak cell density slowly decreased when the percentage of serum in the culture medium is decreased.

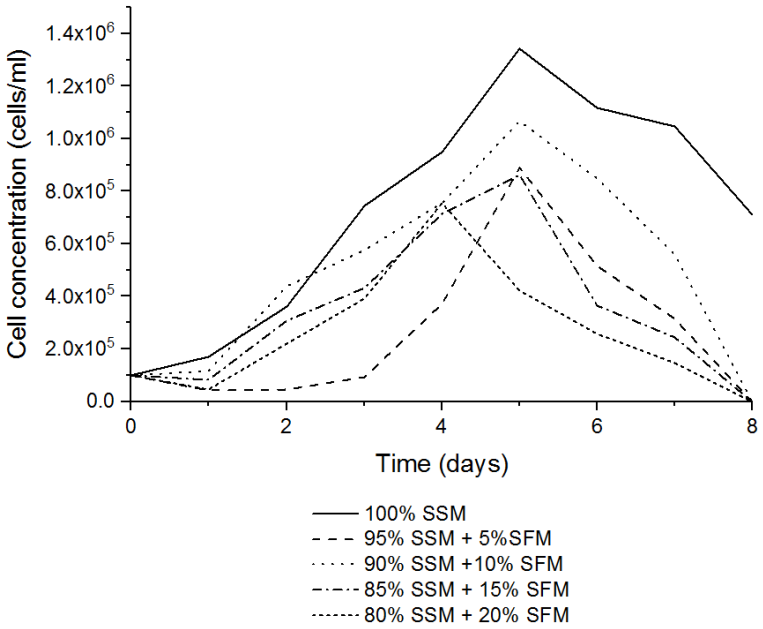


Figure 4: Graph of cell densities in 100 % SSM: 0% SFM, 95% SSM: 5% SFM, 90% SSM: 10% SFM, 85% SSM: 15% SFM and 80% SSM: 20% SFM from day 1 to 8

Table 1: Comparison of maximum cell number with different conditions

Conditions	100% SSM	95% SSM	90% SSM	85% SSM	80% SSM
Maximum cell number	1.34E+06 (Day 5)	8.88E+05 (Day 5)	1.07E+06 (Day 5)	8.62E+05 (Day 5)	7.55E+05 (Day 4)

In the following figures, it was observed that the morphology or growth of the cells that adapted in different conditions to defined media. In the microscopic observations we have witnessed a significant decrease in cell size that correlated with gradual serum reduction adaptations. Figure 4.1 and figure 4.5 were compared between higher percentages of serum with lower percentage of serum where we can observe the drastic changes in the morphology.In 100% SSM, the cells appeared to be much developed, confluent and reached its maximum cell density on day 5. When comparing with 80% SSM the cell were not confluent, can spot large areas without any cell growth in the medium and also on day 4 the cells started to die faster which resulted in the lowest cell density and viability in the medium.

From the overall view of morphology, it is concluded that reduction of serum concentration in the medium composition can cause the cells to die faster.

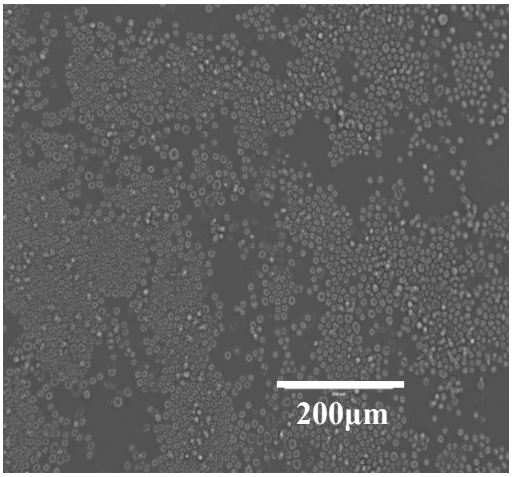


Figure 4.1: Microscopic observation of the cells morphology in 100% SSM on day 5

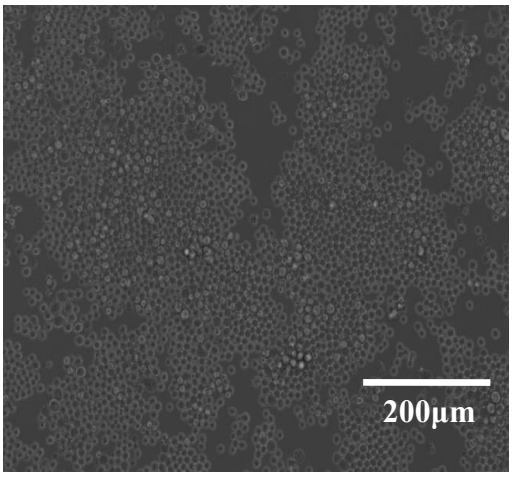


Figure 4.2: Microscopic observation of the cells morphology in 95% SSM: 5% SFM on day 5

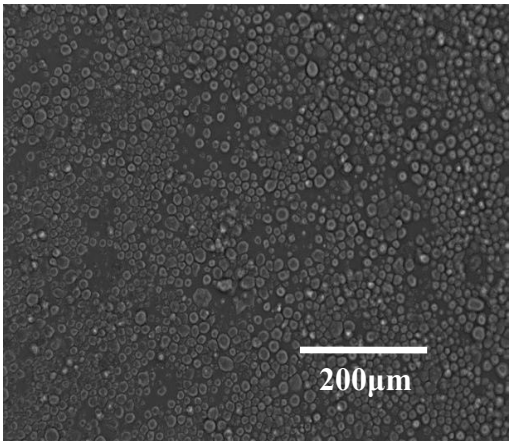


Figure 4.3: Microscopic observation of the cells morphology in 90% SSM: 10% SFM on day 5

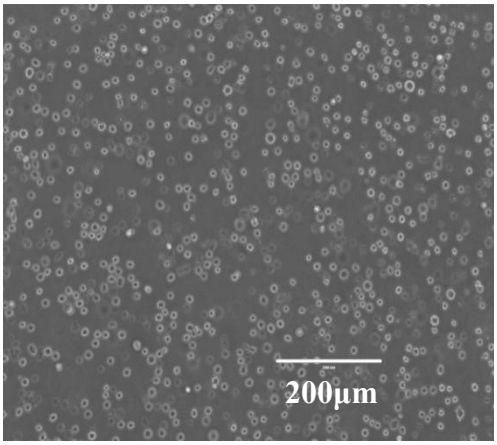


Figure 4.4: Microscopic observation of the cells morphology in 85% SSM: 15% SFM on day 5

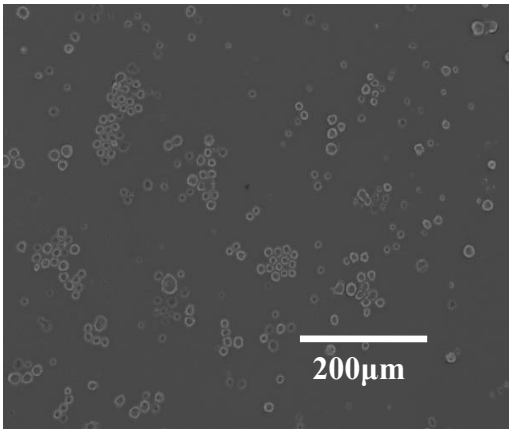


Figure 4.5: Microscopic observation of the cells morphology in 80% SSM: 20% SFM on day 4

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Introduction

In this thesis we have adapted the growth of anti-CEA hybridoma cells in serum-free culture medium (SFM) in five sequential decreasing serum medium compositions. The effects of serum reduction in the culture medium on cell densities, morphology, viability and metabolic profiles was compared and discussed in the previous chapter.

5.2 Conclusion

Based on the results obtained that the cell concentration was significantly reduced as the concentration of serum was decreased (when SSM: SFM ratio increases). It is indicates that the concentration of serum highly influenced the growth rates. High cell density at high serum concentrations is mainly due to the higher concentrations of serum growth stimulatory factors, proteins, vitamins, trace elements, hormones, etc. The presence of serum also influences the amount of glucose uptake and lactate production. Even though many protocols for cellular adaptation to FBS-free conditions have been described, not all the cell lines can be adapted using conventional methods, which are usually based on the progressive removal of serum. Therefore the technique was considered as not compatible for adapting the specific cell line in SFM.

5.3 Recommendations

It is recommended that the composition of the media used need to be a more improved composition with the addition of supplements which is suitable for the anti-CEA hybridoma cells. Then, due to less time consuming, the experiment was not conducted until the percentage of medium reduces to 100% SFM. In next research, it is recommended to conduct the slow adaptation technique from 100% SSM until the composition reaches to 100% SFM. This is because to identify specifically in which compositions the cells can really

performed well. Moreover, perform an additional analysis is suggested to conduct such as glutamine and ammonia concentration analysis. This is because glutamine is an important precursor for the synthesis of purines, pyrimidines, amino sugars, asparagines, and it act as substrate for the TCA cycle and this is reflected in a high rate of utilization. The accumulation of ammonia from glutamine can be decreased by a continuous feed of a low concentration of glutamine into the culture. It also recommended that to perform Enzyme-linked immunosorbent assay (ELISA). This technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. The most essential element in this assay is the detection strategy is a highly specific antibody-antigen interaction.

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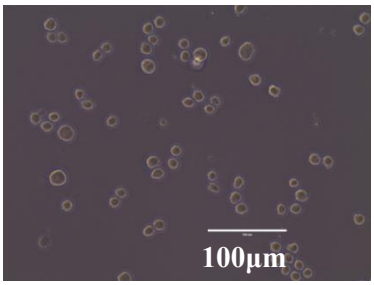
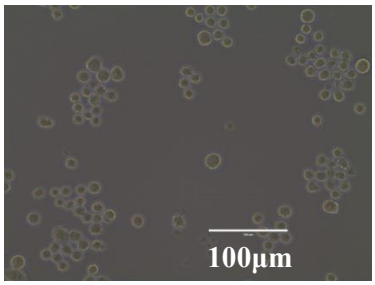
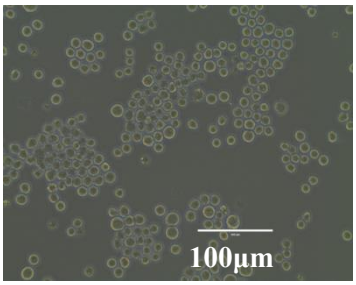
APPENDICES



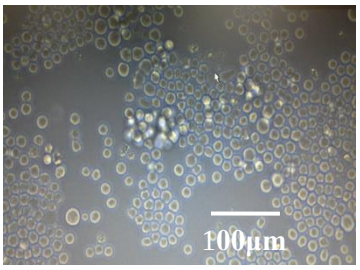
Appendix A: Cells morphology in 100% SSM on day 1



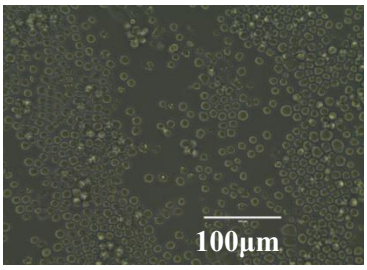
Appendix B: Cells morphology in 100% SSM on day 2



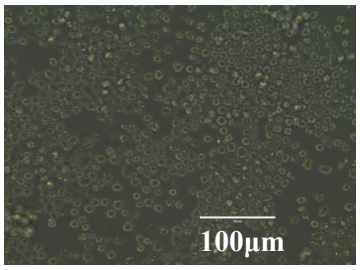
Appendix C: Cells morphology in 100% SSM on day 3



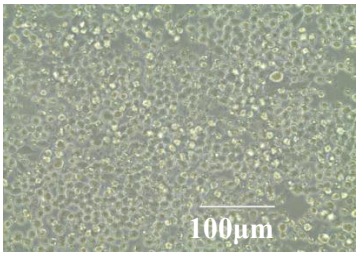
Appendix D: Cells morphology in 100% SSM on day 4



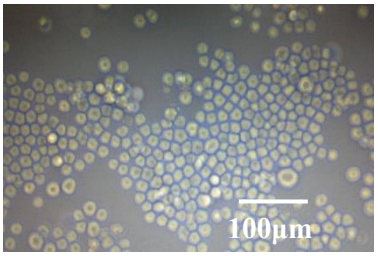
Appendix E: Cells morphology in 100% SSM on day 5



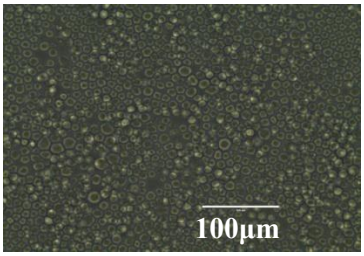
Appendix F: Cells morphology in 100% SSM on day 6



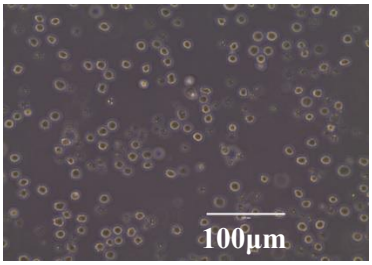
Appendix G: Cells morphology in 100% SSM on day 7



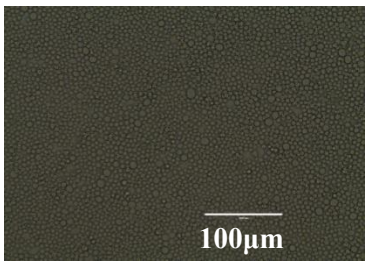
Appendix H: Cells morphology in 100% SSM on day 8



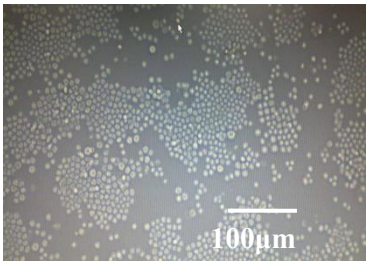
Appendix I: Cells morphology during confluent on day 3



Appendix J: Cells morphology in 100% SSM after cell revival



Appendix K: Cells morphology in subculture on day 2



Appendix L: Cells morphology in 100% SSM on day 7