Process optimization and scale-up for the production of a diagnostic monoclonal antibody against congenital adrenal hyperplasia (CAH)

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ABSTRACT

The objective of this work was to optimize and scale up the suspension culture of the hybridoma 192 cell line for producing a diagnostic monoclonal antibody (MAb) against Congenital Adrenal Hyperplasia (CAH), a rare human genetic disorder. An inexpensive and simple culture method was desired.

The hybridoma 192 had previously been grown only in serum-containing media, mostly in static flasks. Baseline data were obtained in tissue culture flasks (T-flask) with the cells grown in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Cells were first adapted to grow in DMEM with a reduced serum content of 2%. These adapted cells were grown in T-flasks, spinner flasks and a 2 L stirred tank bioreactor, to assess process scalability. Compared to T-flasks and spinner flasks, a high specific MAb production rate could be achieved in the highly controlled conditions of the 2 L bioreactor.

In the next step, the serum content of the media was further reduced to 0.4% by adding inexpensive components (ferric citrate, sodium selenite, zinc sulfate, 2-mercaptoethanol, essential amino acids and ethanolamine) to the medium to replace the functionality of serum. Media design experiments were conducted in T-flasks using a design of experiment methodology to screen components for their effectiveness in substituting serum and supporting the growth of the hybridoma cells. The significant components were then optimized through a central composite design (CCD). The optimized medium was verified after adapting the cells to this new formulation. Of the screened components, only sodium selenite, zinc sulfate and ferric citrate were found to substitute for some of the functions of serum. Serum requirement of the cells was successfully reduced to 0.4% by supplementing the medium with 311.8 μM ferric citrate, 17 nM sodium selenite and 4.5 μM zinc sulfate. With this new formulation, the total cost of the medium was reduced by nearly 80% compared to DMEM supplemented with 5% FBS. The specific growth rate of the cells in this new formulation was comparable to that in the DMEM medium supplemented with 2% FBS. Similarly, the specific MAb production rate in the new medium was comparable to that of DMEM + 2% FBS. The antibody produced in the new formulation could specifically detect its antigen, 17-OHP.

In a further step, amino acids, lysozyme, dimethyl sulfoxide (DMSO) and lipopolysaccharide (LPS) were screened as potential inducers of MAb production in this cell line. An effective inducer (i.e. LPS) was identified, but the level of enhancement of the average specific MAb production rate could not compensate for the high cost of the inducer. Therefore, the inducer was not used in further work.

Using the new low-serum medium, the cell culture was scaled-up to a 2 L bioreactor and the operating parameters were screened with a fractional factorial design. A total of 5 parameters were screened. They were pH, temperature, dissolved oxygen, stirring speed and gas sparging rate. The significant parameters were then optimized with a central composite design. The resulting optimized parameters were verified and used to scale up to a 20 L bioreactor using constant impeller tip speed as the scale up criterion. The performance of cells in the 20 L bioreactor was then evaluated. All 5 operating parameters screened and optimized in the 2 L bioreactor significantly affected the culture performance. The optimum operational condition to maximize the MAb production rate were a sparging rate of 0.09 vvm, a stirring speed of 100 rpm, a pH of 7.4, a temperature of 36.8°C and a dissolved oxygen level of 30% of air saturation. The specific Mab production rate at this optimum point was 0.2730 pg/cell.h and the specific growth rate was 0.0363/h. This was equivalent to a production of 11.941 µg/mL (or 11.9 mg/L) of MAb per batch. By using the constant impeller tip speed scale up criterion, the stirring speed in the 20 L bioreactor was calculated to be 37 rpm. The other operating settings remained the same as in the 2 L bioreactor. There were improvements in the specific growth rate (0.0401/h) and the maximum viable cell density (1.89×10^6 cells/mL) attained by the hybridoma 192 in the 20 L bioreactor. Nevertheless, both the maximum MAb titer (9.782µg/mL) and the average specific MAb production rate (0.1695 pg/cell.h) were slightly reduced relative to the 2 L bioreactor. This was due to the negatively-growth associated production characteristics of the MAb. The product quality was examined through specificity tests and SDS-PAGE. The MAb produced in the 20 L bioreactor was found to be specific to its antigen, 17-OHP.

In conclusion, the production of the diagnostic MAb against CAH was successfully optimized in a newly developed medium that greatly reduced the cost of culture. A successful scale up of the production process to a 20 L bioreactor was demonstrated.

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LIST OF ABBREVIATIONS

[GLN]	Glutamine concentration (mM)
$[GLN]_0$	Initial glutamine concentration (mM)
17OHP	17α-Hydroxyprogesterone
ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ACTH	Adrenocorticotropic hormone
AMM	Ammonia
ANDRD	4-Androstene-3,17-dione
ANOVA	Analysis of variance
BESTR	β-Estradiol
BSA	Bovine serum albumin
CAH	Congenital adrenal hyperplasia
CCD	Central composite design
CHO	Chinese hamster ovary cell line
CV	Coefficient of variation
CYP21	21-Hydroxylase
d	Length of the light path (cm)
DF	DMEM/Ham's F12 medium in a ratio of 1:1 by volume
DHEA	Dehydroepiandrosterone
D_i	Impeller diameter (cm)
DMEM	Dulbecco Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DO	Dissolved oxygen level (% of air saturation)
D_T	Bioreactor diameter (cm)
ELISA	Enzyme Linked Immunosorbent Assay
eRDF	Enhanced RPMI/DMEM/F12 medium in volum ratio of 2:1:1
F12	Ham's F12
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDA	United States Food and Drug Administration
FMOC	9-Fluorenylmethylchloroformate
GC/MS	Gas chromatography tandem mass spectrometry

GIDH	Glutamate dehydrogenase
GLN	Glutamine
GLU	Glucose
HPLC	High Performance Liquid Chromatography
IgG	Immunoglobulin G
IMDM	Iscove's Modified Dulbecco medium
k	Glutamine degradation rate constant (1/h)
k_La	Volumetric oxygen transfer coefficient (1/s)
LAC	Lactate
LC/MS/MS	High-performance liquid chromatography tandem mass spectrometry
LPS	Lipopolysaccharide
LSD	Low serum DMEM
MAb	Monoclonal antibody
MEM	Minimum Eagle's Medium
MW	Molecular weight (g/mol)
MWCO	Molecular weight cut off (Da)
N	Rotational speed (rpm)
NADH	Nicotin-amide-adenine dinucleotide
NM	New medium
OD	Optical density
OPA	Ortho-phthalaldehyde
OUR	Oxygen uptake rate (mmol/(L.h))
PBS	Phosphate buffer saline, pH 7
PEG	Polyethylene glycol
PREG17	17α-Hydroxypregnenolone
PREG5	5-Pregnen-3β-ol-20-one
q	Specific consumption/production rate (mmol/10^9 cells.h)
Q	Volumetric gas flow rate (vvm)
Q^2	Goodness of prediction
r	Average specific MAb production rate (pg/cell.h)
R^2	Goodness of fit
RIA	Radioimmunoassay

RPMI 1640	Roswell Park Memorial Institute 1640 medium
SV	Simple virilising
SW	Salt-wasting
t	Culture period (h)
TEA	Triethylamine
THF	Tetrahydrofuran
TRFS	Time-resolved fluorescence spectroscopy
UP	Ultrapure water
V	Final volume of solution mixture in ammonia assay
v	Sample volume in ammonia assay
$\int X_v.dt$	Viability index (cells.h)
X_v	Viable cell density (cells/mL)
Y	Yield
μ	Specific growth rate (1/h)
μ	Viscosity of the fluid (Pa.s)
τ	Shear stress (Pa)
γ	Shear rate (1/s)
ε	Extinction coefficient of NADH at 340 nm (L/mol.cm)

CHAPTER 1:

INTRODUCTION

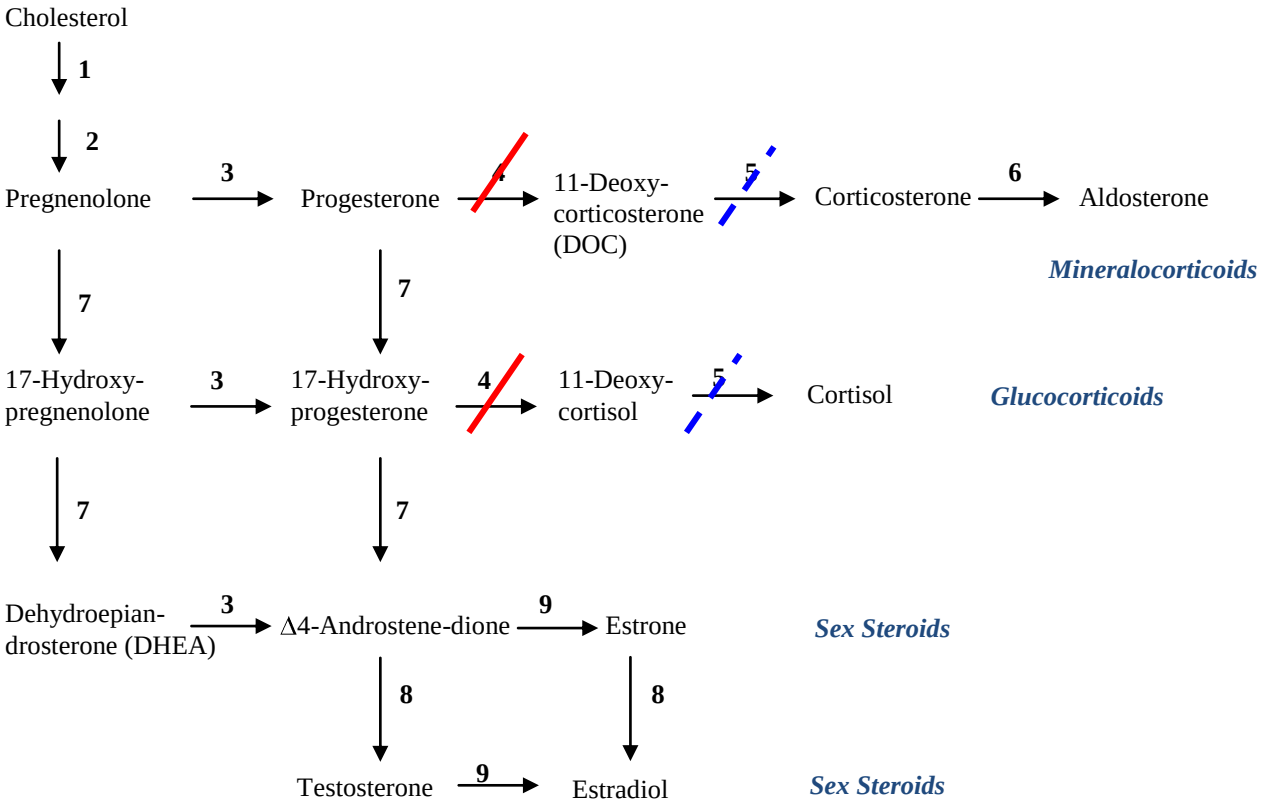
1.1 Background to the study

This work focused on optimization and scale-up of a process for producing a potential diagnostic monoclonal antibody by hybridoma cell culture. The antibody had been developed for diagnosis of congenital adrenal hyperplasia (CAH).

1.1.1 What is CAH?

Congenital adrenal hyperplasia (CAH) is an inherited genetic disorder that is due to the autosomal recessive effects in cortisol biosynthesis. CAH results in glucocorticoid and mineralocorticoid deficiencies and an increased level of adrenocorticotropic hormone (ACTH). ACTH then induces adrenal hyperplasia and overproduction of the steroids that result in various manifestations of the disorder. CAH is associated with defects in enzymes 4 and 5 in steroidal biosynthesis pathway shown in Figure 1.1 (Friedman, 2004).

There are five major types of CAH. Each type is categorized based on which steroids are in excess and which are deficient. The most common type that accounts for 95% of the cases is due to the deficiency of 21-hydroxylase (CYP21). In this condition, there is a failure of 21-hydroxylation and this leads to increased ACTH release from pituitary gland. This in turn causes overproduction of 17-hydroxyprogesterone, progesterone, androstenedione, dehydroepiandrosterone (DHEA) and testosterone (Figure 1.1). 21-Hydroxylase deficiencies can be divided into classic CAH and non-classic CAH.



95% of occurrence

5% of occurrence

Enzyme number	Enzyme
1	Steroidogenic acute regulatory protein
2	Cholesterol side chain cleavage enzyme/desmolase
3	3β-Hydroxylase dehydrogenase
4	21α-Hydroxylase
5	11β-Hydroxylase
6	Corticosterone methyloxidase
7	17α-Hydroxylase/17,20 lyase
8	17β-Hydroxysteroid dehydrogenase
9	Arimatase

Figure 1.1: Pathway of steroid biosynthesis (Friedman, 2004)[1]

[1] Defects in enzyme 4 and 5 are associated with the occurrence of CAH

Classic CAH can usually be diagnosed at birth or during childhood. It can be further divided into the salt-wasting (SW) form and the simple virilising (SV) form. Life-threatening salt-loss crisis, hyponatraemia, hyperkalaemia, dehydration, shock and ambiguous genitalia in females, are the characteristics of SW form. The SV form can be recognized through pseudo-precocious puberty in males and different degree of clitoris hypertrophy and posterior labial fusion in females (Votava *et al.*, 2005). Only one third of classic CAH cases are of SV form; the rest are SW form (Friedman, 2004).

Non-classic or late-onset CAH manifests during or after puberty. It is easier to diagnose in female than male due to the hirsutism and cycle irregularities that are obvious. Even though this type of CAH is not life threatening, it causes accelerated bone maturation that result in a reduced final height (Votava *et al.*, 2005).

1.1.2 Frequency of incidence

Merke and Bornstein (2005) reviewed the various studies involving newborn screening for CAH in 13 countries. The studies covered about 6.5 million newborns and the results showed an overall prevalence of 1:15,000 for classic CAH. Pang and Shook (1997) found the highest prevalence of CAH in two geographically isolated populations, the Yupik Eskimos (1:282) and the La Reunion Island (1:6,071). In Asia, the observed prevalence of CAH ranges from 1:14,822 to 1:16,866 (Chu *et al.*, 2002; Gu *et al.*, 2002; Toyoura *et al.*, 1992). None of the above figures includes data from Africa, Middle East, India, and Pakistan, because such data are not available. This data suggest significant incidence of CAH worldwide. Assuming an average global incidence of 1:15,844 of all forms, a world population of about 6.3 billion, and an average population growth rate of 1.17%, a worldwide annual demand of 73.7 million can be expected for CAH screening kits.

1.1.3 Screening methods available

Several screening methods have been used for CAH since the introduction of newborn screening programs. The currently employed methods include radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), time-resolved fluorescence spectroscopy (TRFS) and tandem mass spectrometry (Török *et al.*, 2003; González *et al.*, 2008; Votava *et al.*, 2005). Some of the newly developed methods include DNA or molecular analysis, gas chromatography tandem mass spectrometry (GC/MS) and high-performance liquid chromatography tandem mass spectrometry (LC/MS/MS) (McCabe and McCabe, 1999; Lai *et al.*, 2002). Table 1.1 compares the various methods. The best assay for use in hospitals is likely to be an ELISA method that is fast, accurate, sensitive, specific and cost effective.

1.1.4 Rationale for using monoclonal antibodies (MAb) for CAH diagnosis

The current ELISA method for diagnosis of CAH is based on polyclonal antibody (González *et al.*, 2008). Use of MAb based assays for detection has many advantages, especially in its specificity that lowers the risk of a false positive diagnosis. In addition, a MAb is a well-defined protein that possesses uniform characteristics as compared to polyclonal antibody. A mouse-mouse hybridoma (hybridoma 192) capable of producing a monoclonal antibody for detecting CAH had been developed by the Physiology Department, Medical Faculty, University of Malaya, Malaysia. The MAb produced by this hybridoma detected the presence of 17-OHP in the samples of serum, to diagnose CAH. This novel hybridoma became the focus of this study for developing a bioreactor-based scalable and inexpensive process for producing the MAb for possible use in clinical testing.

Table 1.1: Advantages and disadvantages of various CAH detection methods

Assay Method	Advantages	Disadvantages
RIA	• Excellent sensitivity (Török *et al.*, 2003)	• Requires expensive equipment • Hazardous in handling radioactive antigen • Requires highly trained personnel
ELISA	• High sensitivity • Ease of handling multiple samples	• Less reliable than RIA (Butler, 1996)
TRFS	• Rapid. • Specific (Votava *et al.*, 2005)	• Low efficiency (Votava *et al.*, 2005)
GC/MS	• Accurate, specific, sensitive (Lai *et al.*, 2002)	• Requires derivatization of sample prior to injection (Lai *et al.*, 2002)
LC/MS/MS	• Rapid, easy, high throughput, accurate, specific and sensitive in component detection (Lai *et al.*, 2002) • Able to handle multiple components (Lai *et al.*, 2002)	• Highly expensive equipment and process (Speiser, 2004) • Requires highly trained personnel (Therrell *et al.*, 1998) • Lengthy in sample preparation (Minutti *et al.*, 2004)
DNA analysis	• Accurate, specific and sensitive (McCabe and McCabe, 1999)	• Costly equipment • Expensive process (van der Kamp and Wit, 2004) • Requires highly trained personnel

1.2 Problem identification

1.2.1 Serum omission from culture medium

Prior to this work, the production of a diagnostic MAb using the hybridoma 192 had been only at the scale of tissue culture flasks in serum containing media. Producing a sufficient quantity of the MAb inexpensively required the development of a bioreactor-based culture process and a serum-free or low-serum medium. Development of such a process for this novel hybridoma was the objectives of this research.

Serum has been conventionally added to the basal cell culture media up to a level of 20% (v/v) in mammalian cell culture research, especially during cultivation of hybridoma cells. Though the role of serum in the culture is not entirely clear, it is believed to contain proteins, hormones and growth factors that support cell growth (Barnes and Sato, 1980). In addition, serum provides protection to the cells from shear damage in stirred culture (Kunas and Papoutsakis, 1990; Michaels *et al.*, 1991) as typically carried out in large-scale bioreactors.

Serum quality varies from batch to batch and this causes a variable production process (Shacter, 1989). In addition, serum is potentially contaminated with etiologic agents, which is undesired for both *in vivo* and *in vitro* use (Freshney, 2005). Therefore, it is important to grow hybridomas in serum- and/or protein-free media to the extent possible. Serum is a poorly defined natural product that contains numerous proteins and undefined components. Its use necessitates complex and expensive downstream purification of the product (Jayme and Smith, 2000). Most importantly, serum is very expensive and contributes nearly 80% to the medium cost (Griffiths, 1986). Exclusion of serum from a culture medium can greatly reduce both the medium cost and the subsequent downstream processing cost.

1.2.2 Requirement of a simple, reliable and consistent small scale batch culture process

Animal cells can be grown in continuous culture or in batch operations. As a result of the absence of equipment such as media and product holding tanks, pumps, etc., the cost of operating a batch system of a similar working volume as a continuous

system is generally much lower (Marquis *et al.*, 1990). The complexity of continuous culture systems and their susceptibility to contamination and mechanical failure (Marquis *et al.*, 1990; Bibila and Robinson, 1995; Tokashiki and Yokohama, 1997; Dalm *et al.*, 2005) is a further disadvantage. Moreover, it is wasteful to discard the valuable serum and nutrient components in the effluent stream (Glacken *et al.*, 1983). Fouling and blockage of the cell retention devices in perfusion culture are other problems that have not been satisfactorily solved (Woodside *et al.*, 1998; Kretzmer, 2002; Voisard *et al.*, 2003). Compared with other operation modes, batch culture has a lower risk of contamination and mechanical failure as it needs no ancillary equipment. Therefore, batch modes of operation will be the primary focus in this study.

Currently, a monoclonal antibody that is able to detect 17OHP is being produced in cyclic-batch culture using CL-6 and CL-1000 culture flask (Integra Bioscience, Switzerland). These are static cultures with no dissolved oxygen and pH control. Thus, the amount of MAb obtained is rather low. In order to prepare a substantial amount of this MAb for clinical trials, a large amount of MAb has to be produced in a short time. Scaling-up the culture using a fully-controlled stirred batch bioreactor appears to be the preferred production option. This option will be examined for the hybridoma 192.

1.2.3 Low producer characteristic of hybridoma cells

Like some other hybridoma cells (Martín-López *et al.*, 2007), hybridoma 192 has a relatively low cell specific production of MAb. Strategies are needed to enhance the cell specific MAb productivity. This is necessary for rapid generation of milligrams of MAb suitable for initial clinical trials. This study will investigate the use of inducer agents to improve MAb protein production and secretion.

1.3 Objective of study

In summary, the objective of this study is to increase the production of MAb against 17OHP, in order to prepare considerable amount of MAb for clinical trial, by using a cheap medium and a simple and scalable process.

1.4 Research strategy

First, the baseline growth and MAb production by the hybridoma 192 was characterized in detail using the culture conditions that had been previously used as standard for this cell line in small scale T-flask culture. Next, attempts were made to greatly reduce or eliminate the serum requirement of the hybridoma 192 without the use of expensive serum substitutes or loss of productivity relative to the best baseline conditions. This helped in reducing the operational costs. Inducers were then screened and the culture medium was further optimized using statistically designed experiments in attempts to enhance the MAb productivity. All of the above was done with a focus on ultimately using the medium in controlled batch cultures in stirred bioreactors. Scalability and operability of the batch production were evaluated at a bioreactor scale of up to 20 L.

1.4.1 Serum reduction and baseline data collection

Hybridoma 192 cell line kindly provided by University of Malaya was originally grown in DMEM supplemented with 5% serum. Therefore, weaning of cells out of serum to the extent possible was required. During this process, two weaning protocols were investigated so as to identify the best one for this cell line. The performance of cells such as growth, antibody production and metabolism were monitored during the processes to assess the effects of serum weaning.

1.4.2 Medium optimization using statistical experimental design

During this study, statistical experimental design was used to identify the potential for further serum reduction and medium enhancement with inexpensive components. This aimed to obtain an optimum medium formulation at the lowest cost possible with minimal supplements, which enhanced both cell growth and MAb production. Effects of inexpensive inducer agents on MAb productivity in an otherwise optimal medium were statistically evaluated.

1.4.3 Operational optimization using statistical experimental design

The technology for large scale production of this novel hybridoma and its corresponding MAb had not been developed. Thus, the culture was scaled-up initially to a 2 L stirred bioreactor. Statistical experimental design was used to screen the important operational factors and optimize them in order to establish the best operating conditions to improve cell growth and MAb production. The culture was then further scaled-up to a 20 L bioreactor using a simple scale-up strategy. The ability of the hybridoma 192 to grow and produce the MAb in a 20 L stirred bioreactor was verified.

CHAPTER 2:

LITERATURE REVIEW

2.1 Monoclonal antibodies and their applications

Polyclonal antibodies are produced by B lymphocytes in the immune system of animals against antigens. Polyclonal antibodies are a mixture of immunoglobulin molecules secreted against specific antigens and recognize the different epitopes on the antigen. In contrast, monoclonal antibodies (MAb) are generated from one type of immune cells, which are clones of a single parent line. Thus, they are monospecific antibodies that are identical and specifically bind only to the antigen that was used to create them. As a consequence MAbs can be used to detect specific antigens and selectively recover them from a mixture. For example, MAbs linked to drugs can be used to specifically target the drug to cancer cells displaying the relevant antigen (Shen and Zhu, 2008). Similarly, the presence of Sudan dyes in food products can be detected using MAbs (Ju *et al.*, 2008). MAbs are used to separate cells displaying the relevant antigen (Kato *et al.*, 2009) and purify proteins from mixtures (Franco *et al.*, 2008; Subramanian, 2002).

2.2 Production of a monoclonal antibody

There are two established methods to produce monoclonal antibodies, i.e. *in vivo* and *in vitro*.

2.2.1 *In vivo* cultivation

In vivo method involves large number of mice, where $10^5 – 10^6$ hybridoma cells are injected intraperitoneally to the mice or rats (Niloperbowo, 1993). The hybridomas grow as tumors or ascites suspended in the peritoneal fluid. The ascites fluid produced by the hybridomas contains the desired monoclonal antibody and can be harvested two

to four weeks after the initial inoculation. This process though simple, requires extensive labor and is not suitable for large scale production. In addition, the ascites fluid is a mixture of the proteins of animal origin and the desired antibody secreted by the hybridoma cells. Thus, purification can be difficult. Most importantly, the ascites technique is painful to the animal and is now considered unethical.

2.2.2 *In vitro* cultivation

In vitro cultivation is ethically acceptable and much more reproducible compared to the *in vivo* method. Furthermore, it is more suitable for large scale production of monoclonal antibodies. Hybridoma cells are grown either as suspension or immobilized cultures in this method. Various types of bioreactors can be used for both suspension and immobilized cells on microcarriers. Examples of common bioreactors include roller bottles, stirred tank bioreactors, air-lift bioreactors and the wave bioreactor (Fong *et al.*, 1997; Burgaski *et al.*, 1989; Jain and Kumar, 2008; Genzel *et al.*, 2006; Singh, 1999; Griffiths, 2000; Chu and Robinson, 2001). *In vitro* cultivation requires more strict operational measures as compared to *in vivo* cultivation method. This is because if contamination of the culture occurs at any stage of the process, the whole operation will need to be terminated. In addition, both medium formulation and physical operating parameters need to be optimized and kept constant to ensure optimum cell growth and monoclonal antibody (MAb) production.

2.2.3 Types of bioreactors

In laboratory scale, animal cells are typically cultured in the tissue culture flask (or T-flask), either reusable or disposable type. A tissue culture flask containing a suitable growth medium and inoculated with the cells is placed in the humidified CO_2 incubator at 37°C with 5-10% CO_2. Generally, pH and dissolved oxygen are not monitored in this case. pH is normally indicated by the color changes of phenol red indicator that is present in the culture medium.

In scaling-up the process to bigger scale, roller bottles are used when culturing adherent cells, while for suspension adapted cells, a spinner flask is more suitable.

There is still no monitoring of pH and dissolved oxygen. Nevertheless, in the spinner flask the cell suspension is stirred, unlike in the static tissue culture flasks. Stirring is inevitably necessary to promote mixing and oxygen transfer when the culture volume increases. When culture volume of greater than 1 L is required, bioreactors with pH and dissolved oxygen controls are generally used.

There are basically three types of large-scale bioreactors that are being used: stirred-tank bioreactors, airlift/bubble column bioreactors and packed-bed/fluidized-bed bioreactors. The most commonly used bioreactor is the stirred-tank. It has a good operating flexibility as the mixing and aeration can be controlled independently and the cells do not need to be immobilized (Backer *et al.*, 1988; Chu and Robinson, 2001; Jain and Kumar, 2008). Nevertheless, for cells which are sensitive to hydrodynamic shear forces, stirred-tank bioreactor with gas sparging for oxygen transfer unavoidably will affect the production performance (Chu and Robinson, 2001; Nienow, 2006). Airlift or bubble column bioreactors provide a reduced mechanical shear stress environment for cell culture (Tokashiki and Yokohama, 1997). However, this type of bioreactor is not suitable if bubbles cause extensive cell damage (Tokashiki and Yokohama, 1997).

Packed-bed or fluidized bed bioreactors are normally used for anchorage-dependent cells, where cells are immobilized on organic or inorganic carriers. A major shortcoming of the packed-bed bioreactor is the difficulty in sampling cells, which makes monitoring of cell growth difficult (Tokashiki and Yokohama, 1997). In addition, there can be problems in inoculating the cells uniformly over the entire packed bed (Tokashiki and Yokohama, 1997). In fluidized bed bioreactors, collisions between carriers can damage the cells (Tokashiki and Yokohama, 1997). A new type of bioreactor the "wave bioreactor" has been introduced (Singh, 1999; Weber *et al.*, 2002; Genzel *et al.*, 2006; Öncül *et al.*, 2010). It consists of a disposable plastic bag which is inflated while in use. Mixing and oxygen supply are achieved by a rocking mechanism. This rocking motion induces waves at the liquid-air interface, improving oxygen transfer and facilitating bulk mixing. Singh (1999) demonstrated that cultivation up to 100 L in this bioreactor was possible with various types of cell lines. This system is most popular in virus production that requires a high level of containment and a relatively small production volume (Weber *et al.*, 2002; Genzel *et al.*, 2006). Even though probes can be inserted to monitor the pH and pO_2, the culture environment is not

fully controlled. Thus, optimized culture conditions are difficult to establish in this type of bioreactor.

2.2.4 Modes of operation

Large-scale cell culture can be run in different modes, depending on the availability of equipment, facilities, product requirements, the amount of product required, the market price and the process feasibility (Kretzmer, 2002). There are generally three basic modes of operation, namely batch, fed-batch and perfusion for animal cell culture. These modes can be further subdivided as discussed in the following sections.

In a batch culture the medium and the cells are charged into the bioreactor and run for a certain period of time. The product is harvested only after the termination of the culture. Cyclic-batch, on the other hand, is a repetition of the batch process. The inoculum for the second cycle comes from the first cycle after a part of the broth has been harvested towards the end of the growth cycle. The cycle is repeated a few times before complete emptying of the bioreactor and cleaning (Shuler and Kargi, 2003).

In fed-batch mode, cells are fed either continuously or intermittently without harvesting in a bioreactor in which the starting volume is low and increases with time. Semi-continuous operation is similar to fed-batch in that the culture is fed intermittently. However, the volume of the culture is constant in this case because a volume of the broth that equals the volume of the feed is harvested before the fresh medium is fed during the growth cycle (Shuler and Kargi, 2003).

Chemostat culture or continuous stirred culture is fed with a fresh nutrient medium continuously. The product, cells and waste metabolites are withdrawn at the same rate from the culture as the rate of addition of the fresh feed. Perfusion culture, in contrast, allows only the product and the waste metabolites to be withdrawn while retaining cells in the bioreactor with various types of devices. Cell retention devices may be installed internally or externally. Examples of internal cell retaining devices are spin-filters, acoustic settlers and gravity settlers (Voisard *et al.*, 2003; Woodside *et al.*,

1998). External cell retaining devices available are the cross-flow filtration modules, hollow fiber modules, centrifuges, controlled shear filtration, vortex-flow filters, spin-filters and hydrocyclones (Voisard *et al.*, 2003; Woodside *et al.*, 1998).

2.2.4.1 Batch culture

The mode of operation that has typically gained approval of the United States Food and Drug Administration (FDA) for production of therapeutic and diagnostic protein is the batch mode (Kretzmer, 2002). The maximum viable cell density that can be achieved in a batch mode is relatively low at up to 1 - 2 $\times$ 10^6 cells/mL (Butler and Jenkins, 1989; Tokashiki and Yokohama, 1997; Woodside *et al.*, 1998). Consequently, batch cultures typically have a low concentration of the target product, which depends highly on the maximum viable cell concentration attained. In batch cultures there are significant changes in nutrient concentrations with time in the culture and toxic metabolites accumulate, which in turn reduces growth and protein production (Woodside *et al.*, 1998; Kretzmer, 2002). Batch production normally experiences inconsistency in output and low final product concentrations (Marquis *et al.*, 1990). This is because of nutrient limitations in batch culture as compared to continuous culture, and also the variation in the final cell yield in the batch culture (Marquis *et al.*, 1990). In addition, the volumetric productivity is the lowest among the various modes of operation available (Woodside *et al.*, 1998).

2.2.4.2 Fed-batch mode

As a result of the accumulation of product, fed-batch culture can achieve high product concentration. Fed-batch operation requires only slight modifications to the equipment used in batch operation. Fed-batch mode has been shown by a number of researchers to prolong cell growth, achieve higher cell density and improve product yield (Bushell *et al.*, 1994; Ljunggren and Häggström, 1994; Reuveny *et al.*, 1986a; Tokashiki and Yokohama, 1997; Gambhir *et al.*, 1999; Lee *et al.*, 2003; Altamirano *et al.*, 2004). Nevertheless, volume throughput per run may be limited by the size of the bioreactor (Dalm *et al.*, 2005). In this mode, the major drawback is the accumulation of

toxic metabolites, which are a major inhibitory factor in achieving high productivity (Feng *et al.*, 2006). Extensive developmental work is necessary to determine the optimal feeding protocols (Marquis *et al.*, 1990) and reduce toxic metabolites accumulation.

2.2.4.3 Perfusion culture

Perfusion culture can produce large volume of a product from a small bioreactor continuously (Bibila and Robinson, 1995; Kretzmer, 2002; Dalm *et al.*, 2005). The viable cell density that can be achieved in this mode of operation is around $10^7 – 10^8$ cells/mL (Tokashiki and Yokohama, 1997; Woodside *et al.*, 1998; Butler, 2005). This inevitably assists in enhancing productivity. Furthermore, because the harvested broth is largely free of cells the load on downstream processing equipment can be reduced. However, product dilution occurs due to continuous medium replenishment (Bibila and Robinson, 1995). One advantage of perfusion systems is a reduced product residence time, which in turn decreases the potential of exposing products to unfavorable culture conditions such as proteases released by the cells (Bibila and Robinson, 1995). Moreover, continuous exposure of cells to fresh nutrients while removing growth-inhibitory metabolites aids in increasing cell growth and productivity (Tokashiki and Yokohama, 1997; Kretzmer, 2002). Some cell lines with unstable cellular productivity are not suitable for use in continuous culture mode because their specific productivities tend to drop if cultured continuously for long periods (Bibila and Robinson, 1995; Marquis *et al.*, 1990).

2.2.4.4 Perfusion culture with controlled feeding

This is a supposedly novel mode of operation that combines the advantages of fed-batch and perfusion cultures. This mode of operation enables high product yield and high volume throughput to be obtained (Yang *et al.*, 2000; Feng *et al.*, 2006). In controlled-fed perfusion culture, the process is run as perfusion culture except that the cell density is controlled at a desired level by including an additional bleed stream. In addition, the feed medium is normally formulated with selected amino acids (Feng *et al.*, 2006) or controlled at a low glucose concentration (Yang *et al.*, 2000). According to

Yang *et al.* (2000), daily productivity of the controlled-fed perfused culture outperformed perfusion culture by about 2-fold and exceeded batch and fed-batch cultures by 10-fold.

2.3 Culture media

A medium is a complex nutrient mixture that is used to grow and maintain the cells *in vitro*. A medium provides essential nutrients for cell growth and generation of the product. Among the nutrients that are necessary for cell growth are sugars, amino acids, fatty acids, vitamins, trace elements and possible other factors. In addition, a medium may contain phosphate buffer and antioxidants (Lambert and Birch, 1985; Freshney, 2005; Mather 1998). Most media also contain phenol red as a pH indicator (Mather, 1998).

2.3.1 Basal media

The most commonly used media for hybridoma cells are Eagle's minimal essential medium (MEM), Dulbecco's modification of Eagle's medium (DMEM), Ham's F12, Roswell Park Memorial Institute 1640 medium (RPMI 1640), Iscove's Modified Dulbecco medium (IMDM) and enhanced RPMI/DMEM/F12 medium (eRDF) in the volume ratio of 2:1:1. The composition of these media is given by Freshney (2005).

2.3.2 Serum-containing media

Conventionally, all culture media have been supplemented with 5 – 20% (vol/vol) serum. Calf, fetal bovine, horse and human sera have been used. Fetal calf serum (FCS; also known as fetal bovine serum, or FBS) is used most commonly. Serum provides the cells with an environment that attempts to reproduce the conditions found *in vivo*.

Serum is a very complex and poorly characterized mixture of proteins and other molecules. Typically, serum contains protein such as albumin, insulin, transferrin and other growth factors that are lacking in the artificial basal media. Serum also acts as a pH buffer (Barnes and Sato, 1980; Maurer, 1986).

Although animal cells have been traditionally grown in serum-containing media, use of serum is associated with many problems. These include the following:

1. Batch-to-batch variability of serum because of variations in genetic, dietary and veterinary history of the animal leads to a variable production process (Shacter, 1989; Jayme and Smith, 2000; Freshney, 2005; van der Valk *et al.*, 2010).
2. The poorly defined nature of serum means that it may contain unwanted components that may interfere with the bioactivity of the product of interest (Barnes and Sato, 1980; Lambert and Birch, 1985; Maurer, 1986).
3. Presence of numerous protein and other undefined components in the serum can present major difficulties with downstream recovery of the product of interest (Jayme and Smith, 2000; Shacter, 1989; Velez *et al.*, 1986; Blasey and Winzer, 1989; van der Valk *et al.*, 2010).
4. Animal serum is potentially contaminated with etiologic agents such as viruses and mycoplasmas (Jayme and Smith, 2000; Freshney, 2005; Blasey and Winzer, 1989; Lambert and Birch, 1985; van der Valk *et al.*, 2010). These agents are difficult to detect with certainty and, therefore, use of serum is undesirable especially when the product of the cell culture is to be used *in vivo*.
5. Serum is generally quite expensive and therefore best avoided to the extent possible (Jayme and Smith, 2000; Freshney, 2005; Velez *et al.*, 1986; Kovář and Franěk, 1986).

In view of the problems mentioned above and the requirement for a consistent and defined medium for producing therapeutic and diagnostic products, extensive research has been carried out in attempts to totally or partially eliminate serum from cell culture media (Chang *et al.*, 1980; Kovář and Franěk, 1986; Blasey and Winzer, 1989; Chua *et al.*, 1994; Martial *et al.*, 1995; Stoll *et al.*, 1996; Radford *et al.*, 1991; Yao *et al.*, 2003; Liu *et al.*, 2001; Parampalli *et al.*, 2007; Huang *et al.*, 2007; Kim *et al.*, 1998 and 1999; Lee *et al.*, 1999).

2.3.3 Serum-free media

In an attempt to eliminate serum from media, Barnes and Sato (1980) found that both insulin and transferrin were stimulatory for every cell type that was studied by the authors. Some of the other cells required supplements in addition to the two already identified (Chang *et al.*, 1980; Murakami *et al.*, 1982; Kovář and Franěk, 1986; Chua *et al.*, 1994; Murakami *et al.*, 1997; Takenouchi and Sugahara, 2003; Müthing *et al.*, 2003).

Using RPMI 1640 as a basal medium with the presence of insulin, transferrin and non-essential amino acids, Chang *et al.* (1980) successfully developed a serum-free medium for their hybridoma cell line (NS-19). They reported that the specific antibody production rate of their hybridoma in this serum-free medium was comparable to that in the serum-containing medium, although the maximum cell density achieved was greatly reduced. In another study, Kovář and Franěk (1986) supplemented RPMI 1640 with insulin, transferrin, ethanolamine, ascorbic acid, hydrocortisone and trace elements in culturing PTF-02 and T3-03 cells. This recipe was sufficient to support cell growth and produce an amount of MAb that was comparable to the level obtained in the serum-containing medium.

Several other basal media have been used to develop serum-free formulations for hybridoma growth. For example, Chua *et al.* (1994) used RPMI 1640, DMEM/F12 and eRDF supplemented with insulin, transferrin, ethanolamine, selenium and bovine serum albumin. The specific growth rates for the hybridoma cell line (2HG11) were lower in all three types of serum-free basal media tested as compared to their serum-supplemented counterparts. Nevertheless, MAb titer obtained in serum-free eRDF medium was the highest (~ 193 mg/L) among the media tested in static culture for about 7 days. Martial *et al.* (1995) employed IMDM/F12 (1:1) as the basal medium and supplemented it with L-glutamine, glucose, iron-saturated human transferrin, polyethylene glycol, ethanolamine, β-mercaptoethanol, ascorbic acid, sodium selenite, essential amino acids, nonessential amino acids, bovine insulin and liposomes. They obtained cell density and MAb titer (25 mg/L) that were comparable to the values seen in the serum-containing medium during continuous culture of VO 208. It appears that a long list of supplements is not necessary for improving cell density and MAb

productivity, as clearly seen for the cases of Kovář and Franěk (1986) and Martial *et al.* (1995).

Proprietary serum-free media such as HB101 (Franco *et al.*, 1999), HB GRO (deZengotita *et al.*, 2002a and 2002b), ASF103 and ASF104 (Terada *et al.*, 2002) have also been used to culture hybridoma cells for producing monoclonal antibodies. The maximum cell density and MAb productivity achieved were comparable to the values observed with the corresponding serum-supplemented media. Different cell lines appear to require different recipes of serum-free media, and the recipe varies with the culture process and conditions (Maurer, 1986; van der Valk *et al.*, 2010). Consequently, a case-by-case optimization of the formulation is important for developing serum-free media for use in large-scale culture.

2.3.4 Protein-free media

As stated in the Section 2.3.2, elimination of serum can possibly reduce the cost of large-scale cell culture. However, if serum is simply replaced with other expensive proteins, hormones and growth factors as mentioned in Section 2.3.3, the resulting serum-free medium may not reduce production costs (Lambert and Birch, 1985; Maurer, 1986; Shacter, 1989; Jayme and Smith, 2000). A long list of supplements further means an extended media preparation time (Shacter, 1989) and labor.

Moreover, supplementing a medium with proteins like insulin, transferrin and serum albumin can interfere with the recovery and purification of the secreted target protein (Nagira *et al.*, 1995; Stoll *et al.*, 1996; Jayme and Smith, 2000). This is particularly so when the target products are monoclonal antibodies that are usually produced in low concentrations compared to the concentrations of the protein additives such as insulin, transferrin and bovine serum albumin.

Serum and other animal-sourced protein are potentially contaminated with difficult-to-detect etiologic agents (Freshney, 2005; Shacter, 1989; Jayme and Smith, 2000; Hesse and Wagner, 2000; van der Valk *et al.*, 2010). This is another reason for elimination of serum and other animal-derived proteins from culture media especially if

the objective is to produce a human therapeutic agent. This consideration is of course not of significance for *in vitro* diagnostic MAbs, but the high cost of serum is.

Blasey and Winzer (1989) worked towards eliminating bovine serum albumin from their medium. They attempted to replace serum's function as a fatty acid carrier with 0.1% (w/v) polyethylene glycol, PEG. The medium consisted of IMDM/F12 (1:1) supplemented with iron saturated human transferrin and bovine insulin. Initial experiments in batch culture were unsatisfactory as the MAb production ceased upon the cells reaching the stationary phase. A fed-batch strategy was more successful. A solution containing glucose, PEG and some MEM amino acid was used for feeding once the viable cell concentration had reached about 1×10^6 cells/mL. This caused a resumption of MAb production. As a result, a final cell density of 3×10^6 cells/mL and a final MAb titer of 187 mg/L were achieved. In another study (Chua *et al.*, 1994), BSA was totally eliminated from the serum-free medium. This increased the maximum cell density attained, but the MAb level was not increased relative to control (Chua *et al.*, 1994). Butler *et al.* (1999) found that linoleic acid could replace the function of bovine serum albumin in protecting cells under shear damaging conditions in the medium. The serum-free media of Blasey and Winzer (1989), Chua *et al.* (1994) and Butler *et al.* (1999) did contain insulin and/or transferrin in their formulations.

Wong *et al.* (2004) successfully replaced the insulin in their serum-free medium with 1.5 mg/L of $ZnSO_4.7H_2O$. They were able to obtain 110 mg/L of antibody after 90 h of culture in an insulin-free Zn-supplemented medium. Iron compounds such as $FeSO_4$ (Shinmoto *et al.*, 1991) and ferric citrate (Kovář and Franěk, 1987; Nagira *et al.*, 1995; Franěk and Fussenegger, 2005) are able to successfully replace the iron-transport protein transferrin. Both the iron salts produced maximum cell concentration and MAb productivity results comparable to those obtained in transferrin-supplemented serum-free media. Clearly, development of a simple protein-free medium such as the one used by Wong *et al.* (2004) is possible without sacrificing productivity in at least some cell lines.

Several researchers have developed protein-free media by starting with a proprietary basal medium formulation such as FMX-Turbodoma. Stoll *et al.* (1996) supplemented this basal medium with gentamycin sulfate, glucose, glutamine, Pluronic

acid F-68 and several amino acids. Ducommun *et al.* (2001) supplemented FMX-Turbodoma with retinol acetate, ergocalciferol, D-alpha-tocopherol acetate, menadione sodium bisulfite, D-biotin, Ca-pantothenate, choline chloride, cyanocobalamine, folic acid, myo-inositol, niacinamide, pyridoxine HCl, riboflavine and thiamine HCl. In both cases, supplements significantly improved cell growth, prolonged the stationary phase before the onset of decline and increased the MAb production rate. In view of the above, in many cases it is possible to totally eliminate protein from culture media by providing appropriate supplements.

2.4 Factors affecting cell growth, cell metabolism and antibody production

2.4.1 Temperature

In order to simulate normal body environment, mammalian cell cultivations are typically run at 37°C. For culture in T-flasks, culture flasks and roller bottles, the temperature is controlled via heated air in a CO_2 incubator. In bioreactors, temperature is normally maintained through a re-circulating water jacket around the vessel or by a hot air bath.

Generally, optimal growth temperature for mammalian cells is 33° to 38°C (Jorjani and Ozturk, 1999). Several researchers attempted to optimize the culture process by varying the culture temperature (Reuveny *et al.*, 1986b; Sureshkumar and Mutharasan, 1991; Borth *et al.*, 1992; Bloemkolk *et al.*, 1992; Barnabé and Butler, 1994; Hovey *et al.*, 1994; Chuppa *et al.*, 1997; Yoon *et al.*, 2004; Clark *et al.*, 2004). The temperature range studied ranged from 29°C to 42°C. However, no growth was observed at both 29°C and 42°C (Sureshkumar and Mutharasan, 1991). Optimum growth at 33°C was reported for hybridoma cells (HB-32) in serum-supplemented static culture (Sureshkumar and Mutharasan, 1991). A lower optimal growth temperature (31°C) was reported by Reuveny *et al.* (1986b) for their hybridoma VIII H-8 in serum-containing static culture. In contrast, Bloemkolk *et al.* (1992) observed optimum growth at 37°C for the static culture and 34°C for the agitated culture for hybridoma S4B6 in a serum-containing medium. On the other hand, Barnabé and Butler (1994) who cultured their hybridoma CC9C10 in a serum-free medium observed similar growth rates and

maximum viable cell counts at 33°C and 37°C. All the reported studies above showed extended culture viability at lower temperature. Hence, it appears that different cell lines show different optimum growth temperature, which is limited to the range of 33° to 37°C.

Sureshkumar and Mutharasan (1991) reported an increased cell metabolism when the culture temperature was increased. The glucose and glutamine uptake rates, and lactate and ammonium consumption rates, increased as the temperature increased. Similar observations were reported by Barnabé and Butler (1994). Nevertheless, Barnabé and Butler (1994) reported a constant yield of lactate from glucose at all the temperatures studied. While Sureshkumar and Mutharasan (1991) found an increased lactate yield from glucose as the temperature increased. These differences in observations might be due to the different culture media used. Sureshkumar and Mutharasan (1991) used serum-supplemented medium, but Barnabé and Butler (1994) used a serum-free medium.

Different cell lines seem to attain maximum antibody titers at different temperature. Hybridoma HB-32 cultured in serum-containing static culture achieved maximum antibody titer of 56 mg/L at 35°C (Sureshkumar and Mutharasan, 1991). For hybridoma S4B6, the highest MAb yield was obtained at 37°C in serum-containing static culture with a value of about 28.7 mg/L. Whereas in agitated culture, the MAb yields at 34° and 37°C were similar (Bloemkolk *et al.*, 1992). In contrast, Reuveny *et al.* (1986b) observed an increase in maximum MAb level attained with increasing temperature and the highest MAb titer was achieved at 37°C (227 μg/mL) for hybridoma VIII H-8 in static serum-supplemented medium. For hybridoma CC9C10 in serum-free medium, Barnabé and Butler (1994) reported a similar value of the maximum titer (136 μg/mL) at 37° and 39°C. In other studies, specific MAb production rate has been found to be constant at a particular temperature, but its value increased with increasing temperature in the temperature range studied (Sureshkumar and Mutharasan, 1991; Reuveny *et al.*, 1986b; Barnabé and Butler, 1994). As reported by Borth *et al.* (1992), specific antibody production rate of hybridoma LC4 cells showed a trend towards increased productivity at high growth rates. Conversely, specific antibody production rate of hybridoma P24 remained unchanged in the range of

temperature studied (Borth *et al.*, 1992). A similar observation was reported by Bloemkolk *et al.* (1992), where the specific MAb production rate was constant (~ 1.0 pg/cell.h) for each batch experiment, regardless of temperature. It appears that specific MAb production rate is not affected by the temperature, but the maximum MAb titer is.

Owing to the different optimum temperature for growth and MAb production, several researchers attempted to optimize the productivity by temperature shift. Cells were first cultured at the optimum temperature for growth in order to achieve comparable amount of cells. Temperature was then shifted to a value that was optimal for MAb production. In such a temperature shift study, a comparable total MAb titer was obtained by Sureshkumar and Mutharasan (1991) with and without the shift. Similarly, Barnabé and Butler (1994) and Reuveny *et al.* (1986b) found that no significant improvement in MAb yield were obtained with the temperature shift strategy.

One study reported that cells had better resistance to shear at lower temperature (Ludwig *et al.*, 1992). Lower culture temperature was also shown to reduce proteolytic activity, which is detrimental to the product quality (Chuppa *et al.*, 1997) and stability. A reduction in oxygen consumption rate following a decrease in culture temperature was also observed (Chuppa *et al.*, 1997; Jorjani and Ozturk, 1999). This suggests that a relatively high cell density can be supported in an oxygen transfer limited bioreactor by reducing the culture temperature (Chuppa *et al.*, 1997).

In summary, it appears that the response of a cell line to different culture temperatures is dependent on the type of cell line, the medium type and the culture conditions.

2.4.2 pH

Culture pH is one of the parameters that needs to be optimized in fermentation processes (Tokashiki and Yokohama, 1997). Physiological pH range for animal cells is between 6.5 and 7.5 (Oeggerli *et al.*, 1995), but the optimum pH for cultivation is 6.7 – 7.4 (Wagner, 1997).

pH in the culture is normally measured by a pH electrode in the bioreactor, while in the culture flask the pH value is approximately indicated by the change in color of the medium that contains a pH-sensitive dye such as phenol red. pH value in the culture can be controlled by varying the CO_2 content in the reactor aeration gas and by adding either $NaHCO_3$ or NaOH to the culture broth, in response to the pH signal. Addition of alkali or acid to control pH can change the osmotic pressure (Oeggerli *et al.*, 1995) in the medium and this may be harmful to the cells. Therefore, pH control by adjusting the gas composition is preferred.

McQueen and Bailey (1990) reported that cell growth rate for hybridoma 131 cultured in a stirred bioreactor varied insignificantly over the pH range of 6.8 – 7.6, but the maximum cell density was lower at pH 6.8. In contrast, Schmid *et al.* (1990) obtained the highest maximum cell density at a low pH even though the cell growth rate was similar at all pH values studied in the static culture of hybridoma AB2-143.2. Ozturk and Palsson (1991a), on the other hand, observed an optimum growth at pH 7.2 for hybridoma 167.4G5.3. Above and below pH 7.2, cell growth rate was reduced. Miller *et al.* (1988a) culturing their hybridoma AB2-143.2 in a continuous mode observed an optimal pH range of 7.1 – 7.4 and a dramatic drop in viable cell density at pH 6.8. These different observations might be due to the different cell lines and media used, and also differences in the modes of operation and methods of study.

In several studies, as the culture pH decreased, the glucose consumption and lactate production were decreased (McQueen and Bailey, 1990; Yoon *et al.*, 2004; Ozturk and Palsson, 1991a; Miller *et al.*, 1988a; Schmid *et al.*, 1990). The yield of lactate on glucose ($Y_{Lac/Glu}$) was also generally reported to decrease with decreasing pH (Ozturk and Palsson, 1991a; Schmid *et al.*, 1990; Yoon *et al.*, 2004; Miller *et al.*, 1988a), but McQueen and Bailey (1990) reported increased values of $Y_{Lac/Glu}$ with decreasing pH.

Different observations have been reported for the effect of pH changes on glutamine metabolism. Ozturk and Palsson (1991a) reported a decreased specific glutamine consumption rate and ammonium production rate as the pH decreased. Both rates were no longer affected by pH below pH 7.2, but yield of ammonium on glutamine ($Y_{Amm/Glm}$) continued to increase with decreasing pH. In contrast, Miller *et al.*

(1988a) observed an increase in both glutamine consumption and $Y_{Amm/Glm}$ as the pH decreased, although the ammonium production rate was fairly constant. Conversely, glutamine consumption, ammonium production and $Y_{Amm/Glm}$ all remained approximately constant in the work of McQueen and Bailey (1990). The obvious difference in these three studies was the different cell lines that were used.

In another study, amino acids consumption rates were generally higher at higher pH values (Schmid *et al.*, 1990), but Ozturk and Palsson (1991a) observed a maximum in amino acids consumption rate at pH 7.2. Generally, as the pH is increased within the physiological range, cell metabolism increases.

Effect of pH on the specific antibody production rate has been studied by several researchers. McQueen and Bailey (1990) observed no influence of pH on the specific antibody production rate. In contrast, Ozturk and Palsson (1991a) and Miller *et al.* (1988a) reported an increased specific antibody production rate under acidic conditions. Miller *et al.* (1988a) reported a similar increase at high pH value (i.e. alkaline conditions).

As can be seen from the studies above, maintaining the culture pH at a value lower than the normally used pH of 7.0 may have benefits. As the cell metabolism decreases with decreasing pH and glutamine decomposition rate is reduced at lower pH values (Ozturk and Palsson, 1990b), possible savings in the cost of medium may be attained at low pH especially if the culture is run in the perfusion mode. Furthermore, enhanced culture longevity was reported at low pH values (Schmid *et al.*, 1990; Yoon *et al.*, 2004). Thus, a low pH has other possible benefits in being able to raise MAb titers, as antibody production is dependent on the fraction of viable cells in the culture (Renard *et al.*, 1988). Moreover, high specific antibody production rates may be obtained at low pH as mentioned above.

2.4.3 Dissolved oxygen content

Mammalian cells require oxygen. Oxygen is required in metabolism, cell growth and even MAb production. Therefore, oxygen is one of the important nutrients for animal cells. However, oxygen has a low solubility in aqueous solutions, only about 0.43 mmol/L at 37°C with air aeration (Wagner, 1997). Consequently, provision of sufficient amount of oxygen to the cells becomes a major challenge in large-scale cultivation of animal cells. In stirred bioreactor, due to the shear sensitivity of animal cells, agitation rate and oxygen transfer are usually limited (Ozturk and Palsson, 1990a; Oeggerli *et al.*, 1995). Conversely, diffusional resistance limits the oxygen transfer in high cell density immobilized bioreactor. Hence, it is important to understand the behavior of cells under oxygen limited conditions in order to optimize the production process.

The effects of dissolved oxygen (DO) on animal cell growth, cell metabolism and MAb production have been studied extensively (Ozturk and Palsson, 1990a, 1991a; Jan *et al.*, 1997; Ogawa *et al.*, 1992; Boraston *et al.*, 1984; Miller *et al.*, 1987, 1988b). Anaerobic conditions can severely depress the cell growth (Ozturk and Palsson, 1991a) but cells do grow under low levels of oxygen. For example, Brosemer and Rutter (1961) showed that their particular cell lines were able to grow at oxygen concentration levels as low as 0.5% of air saturation. Despite low growth rate, cells viability was higher than typically seen under normal higher oxygen conditions. Similar observations have been reported by Ozturk and Palsson (1990a) and Philips *et al.* (1987). On the other hand, oxidative damage has been observed to inhibit cell growth at high oxygen concentrations (Brosemer and Rutter, 1961; Kilburn and Webb, 1968; Philips *et al.*, 1987; Ozturk and Palsson, 1991a; Jan *et al.*, 1997). The cells were able to recover from this inhibition after a short period of exposure once the dissolved oxygen level was brought down to the normal range (Brosemer and Rutter, 1961; Ozturk and Palsson, 1990a; Jan *et al.*, 1997). In general, cells are well tolerant of a wide range of dissolved oxygen (DO) concentrations between 10 and 100% of air saturation (Fleischaker and Sinskey, 1981; Ozturk and Palsson, 1990a and 1991a; Jan *et al.*, 1997; Wagner, 1997). Jan *et al.* (1997) have pointed out that at least some cells are able to grow at a DO level of 125% of air saturation, with prior adaptation in continuous culture.

A number of investigators have studied the effects of oxygen on hybridoma cell growth (Phillips *et al.*, 1987; Reuveny *et al.*, 1987; Ozturk and Palsson, 1990a and 1991a; Ogawa *et al.*, 1992; Jan *et al.*, 1997). Considerable variation has been reported on the optimum DO value for hybridoma growth. Reuveny *et al.* (1986a) and Macmillan *et al.* (1987) reported a maximum specific growth rate at 60% DO for hybridoma VIII H-8 in stirred reactors. While Ozturk and Palsson (1990a) reported a maximum specific growth rate at 35% DO in continuous culture and 50% DO in batch culture (Ozturk and Palsson, 1991a) for the murine hybridoma cell line 167.4G5.3. It appears that each cell line has a different optimal DO concentration and the optimal level of DO depends on the culture conditions as well. Generally, a DO level of around 50% of air saturation is considered optimal for growth of most hybridomas. Specific growth rates are generally constant in the DO level range of 10% - 80% of air saturation and decline at high and low DO concentrations (Ozturk and Palsson, 1991a; Ogawa *et al.*, 1992; Jan *et al.*, 1997).

Mammalian cell metabolism is significantly influenced by dissolved oxygen. Both specific glucose consumption rate and specific lactate production rate were observed to be at their lowest values at 10% DO in batch mode by Ozturk and Palsson (1991a). In continuous culture, Reuveny *et al.* (1986a) showed an increase in specific glucose consumption rate and specific lactate production rate in the DO range of 25-75% air saturation. In contrast, both rates were found to be constant in continuous culture by Ozturk and Palsson (1990a) above 1.2% DO and increased sharply with decreasing of DO from 1.2% to 0%. A similar trend was reported by Brosemer and Rutter (1961) for AH cells. Jan *et al.* (1997) studied the effect of DO concentration above 10% air saturation in serum-free medium for hybridoma culture and reported an increase in both specific glucose consumption and specific lactate production rates at high oxygen concentrations. However, Ogawa *et al.* (1992) observed an opposite trend, where the murine hybridoma 16-3F showed a decreased specific glucose consumption rate when the DO level was increased in a serum-free medium. No changes of specific glucose consumption rate with DO were observed in the serum-containing medium (Ogawa *et al.*, 1992). On the other hand, an increase in yield of lactate from glucose with an increase of dissolved oxygen concentration level was observed in a serum-free medium in continuous culture (Jan *et al.*, 1997). Ozturk and Palsson (1991a) who

cultured their hybridoma in a serum-containing medium, reported a decrease in the yield of lactate when DO level increased in batch culture. In contrast, in continuous culture that used the same cell line and culture medium, the yield of lactate from glucose was constant (~1.34 mol/mol) for the range of 5% to 100% DO, but increased to about 1.86 mol/mol at DO levels below 5%. The increase of both specific glucose consumption rate and specific lactate production rate under anaerobic condition were explained by the Pasteur effect (Ozturk and Palsson, 1990a and 1991), where cells use more glucose for each unit of energy production through the glycolysis pathway.

In batch culture, an increased glutamine uptake rate was observed for very high (100%) and very low (1%) oxygen concentrations (Ozturk and Palsson, 1991a). The minimum glutamine uptake rate was at a DO level of 10%. A similar increase in glutamine consumption rate was reported in continuous culture at both high and low DO levels (Ozturk and Palsson, 1990a; Jan *et al.*, 1997). Ammonium production rate followed the trend of glutamine uptake rate in all cases in the similar ranges of oxygen concentrations. Furthermore, the yield of ammonia from glutamine was constant in all cases reported (Ozturk and Palsson, 1990a, 1991a; Jan *et al.*, 1997).

Not many studies have been done on the effect of dissolved oxygen on amino acid metabolism. Jan *et al.* (1997) reported no significant changes of amino acid metabolism at normal oxygen concentration compared to a significant increase in consumption of all amino acids at 125% DO level. Amino acids metabolism was found to parallel the trend of glutamine consumption rate in both batch and continuous modes studied by Ozturk and Palsson (1990a, 1991a). However, the amino acid consumption rate was one order of magnitude lower than the glutamine uptake rate.

Oxygen uptake rate (OUR) measures the ability of a cell line to consume the oxygen that is being supplied. Therefore, it is important to know the effect of variation in DO concentration on the OUR. As quoted by Lavery and Nienow (1987), oxygen uptake rates for mammalian cell lines are in the range of $2 - 10 \times 10^{-12}$ g/cell.h, which is very much lower than that of Streptococci bacteria (oxygen uptake rate in the range of $1.46 - 2.33 \times 10^{-3}$ g/cell.h). Oxygen uptake rate did not change with DO concentration above 10% air saturation (Fleischaker and Sinskey, 1981; Ozturk and Palsson, 1990a,

1991a; Jan *et al.*, 1997). However, oxygen uptake rate decreased in oxygen-limited conditions and became zero at 0% DO (Ozturk and Palsson, 1990a).

Oxygen concentration also influences antibody production. Different DO levels result in different cell concentrations and this in turn leads to different antibody concentrations as the antibody production strongly depends on the viable cell concentration. In batch and continuous cultures, a maximum antibody production at around 25% - 30% air saturation was reported (Ozturk and Palsson, 1990a, 1991a; Reuveny *et al.*, 1986a). Phillips *et al.* (1987) reported a maximum antibody production at 50% DO. Specific antibody production rate showed no significant variation with the changes in DO concentration above 10% (Ozturk and Palsson, 1991a; Jan *et al.*, 1997). In contrast, Ogawa *et al.* (1992) reported a decreased specific antibody production rate with decreased levels of DO in a serum-free medium as compared to a constant value in a serum-containing medium. While the specific antibody production rate was reported to increase slightly at DO below 1.25% of air saturation by Ozturk and Palsson (1990a). Therefore, it can be concluded that specific antibody production is constant at normal oxygen levels, but the optimal DO for antibody production depends on the cell line. Furthermore, the culture medium that is employed influences the optimal DO level. Generally, the maximum antibody titer that is obtained in a culture is strongly dependent on the ability to maintain high cell viability over an extended period. Most researchers (Reuveny *et al.*, 1986b; Renard *et al.*, 1988; Miller *et al.*, 1988a; Lee *et al.*, 1988) therefore suggest the use of conditions that promote a high cell viability as opposed to conditions that promote rapid growth, to obtain high MAb titers.

2.4.3.1 Dissolved oxygen concentration measurement

On-line measurement of dissolved oxygen typically uses dissolved oxygen (DO) electrodes. There are two types of electrodes, galvanic and polarographic (Doran, 1995). Both types measure the partial pressure of oxygen. Thus, oxygen solubility in the medium at the pressure and temperature of measurement must be determined in order to convert the reading to a dissolved oxygen value. In using DO electrodes for measuring oxygen uptake rate (OUR), it is important to check for the electrode response time, as a slow response time and/or high oxygen consumption by the electrode will increase

errors in the calculation of OUR (Jorjani and Ozturk, 1999). To measure the electrode response time, the electrode that is at steady state in a medium saturated with nitrogen is transferred instantaneously to a well-agitated medium saturated with air. The time taken for the probe to indicate 63% of the total change in DO level is the electrode response time. On-line measurements of oxygen mass transfer coefficient and oxygen uptake rate using dissolved oxygen probes will be most appropriate for the purposes of this work.

2.4.3.2 Methods of controlling dissolved oxygen

In aerated bioreactors for suspension culture of animal cells, control of dissolved oxygen relies on two main methods: (1) increase in aeration rate and/or the agitation speed of the impeller, to enhance mass transfer of oxygen; and (2) increase in oxygen concentration in the aeration gas, to enhance the mass transfer driving force. Both these methods are widely used, but the maximum permissible aeration rates and agitation speeds are limited by what the cells can withstand without being damaged (Oeggerli *et al.*, 1995). Use of pure oxygen and oxygen-supplemented air can be expensive.

Solubility of oxygen in water is very low, and it is even lower in culture media (Robinson and Cooper, 1970; Slininger *et al.*, 1989; Popović *et al.*, 1979; Schneider and Moser, 1984; Reynafarje *et al.*, 1985; Ho *et al.*, 1986; Garcia-Ochoa and Gomez, 2005). Therefore, it is necessary to supply the medium with oxygen continuously. Mammalian cells have a relatively large size as compared with microbial cells. Furthermore, they have no cell walls to protect them. As a result, methods of supplying oxygen that are typically used in microbial system are difficult to apply, mainly because cells are sensitive to shear forces (Glacken *et al.*, 1983), that inevitably accompany aeration and agitation.

There are generally three methods to supply oxygen to the culture. These are surface aeration, air sparging and bubble-free aeration. In surface aeration the aeration gas (oxygen or air) flows through the headspace of the bioreactor above the level of the liquid. This type of aeration is feasible at small scale; for example in shakes flasks, spinner flasks and bioreactors smaller than 5 L (Humphrey, 1998). When the scale of the reactor exceeds 5 L, the surface-area-to-volume ratio in a typical stirred tank

becomes such that oxygen demand cannot be met for typical cells at the kind of cell concentration that would normally occur.

In sparged or submerged aeration, air or oxygen is supplied through a sparger directly into the culture medium. The sparger head, often a single hole, is normally placed right below the impeller at the axis of the bioreactor (Chisti and Moo-Young, 1999). Together with appropriate impellers, such as marine impeller and axial flow impeller (Humphrey, 1998), air sparging is more effective in transferring oxygen into the culture as compared to surface aeration. Nevertheless, bubble formation through sparging has been reported to cause cell damage (Kilburn and Webb, 1968; Glacken *et al.*, 1983). Furthermore, rupture of gas bubbles at the surface of the culture is also known to be a major cause of cell damage (Chisti, 2000). Additionally, sparging causes excessive foaming that can clog the air exhaust filter (Glacken *et al.*, 1983). To protect cells from the damaging effects of sparging, additives such as Pluronic F68 (Kilburn and Webb, 1968; Gardner *et al.*, 1990; Zhang *et al.*, 1992; Jordan *et al.*,1994), PEG (polyethylene glycol) (Papoutsakis, 1991a) and linoleic acid (Butler *et al.*, 1999) are added to the culture media. Foaming associated with sparging is due to the presence of high concentrations of proteins in serum containing media (Glacken *et al.*, 1983); therefore, serum-free and protein-free media supplemented with proper protective agents are better suited to air sparging than are serum-containing media. Sparged aeration is the most common method of supplying oxygen to large submerged culture bioreactors (Chisti, 2000; Merchuk, 1991).

In view of the above-mentioned problems, methods have been developed for supplying oxygen by bubble-free aeration. In one variant of bubble-free aeration, gas permeable membranes are used to provide oxygen (Fleischaker and Sinskey, 1981; Wagner and Lehmann, 1988; Beeton *et al.*, 1991; Schneider *et al.*, 1995). This method uses hydrophobic porous membranes through which air diffuses into the liquid medium without bubbling. However, long tubing is required in large scale operations to provide sufficient surface area and this is expensive. The tubes have a finite life and their presence in the reactor can interfere with mixing. An alternative is the use of perfluorocarbons in which the oxygen has a high solubility (Lowe *et al.*, 1998; Hamamoto *et al.*, 1987). Finally, there are also perfusion loop reactors where the cell-free culture medium is circulated and oxygenated through an external loop (Burgaski *et*

al., 1989). The drawback of this method is that a high circulation rate is required in large scale operations and this can cause excessive foaming and cell damage.

2.4.4 Shear effect

Animal cells are quite susceptible to shear, mainly due to their lack of a protective cell wall and their relatively large size (~10-20 μm). In large-scale cultivation of suspended animal cells, the most typical method used is stirred bioreactor (van der Pol and Tramper, 1998). The advantages of using this bioreactor are the uniform culture conditions, superior process control and monitoring of process parameters, ease of scaling-up from 1- to 1000-litre, and use of existing industrial capacity (van der Pol and Tramper, 1998; Papoutsakis, 1991b; Kunas and Papoutsakis, 1990b; Wu, 1995; Butler *et al.*, 1999).

Mixing in stirred bioreactors is done by mechanical agitation, which inevitably imposes certain hydrodynamic stress on cells. Turbulent flow in bulk liquid has been shown to cause only slight damage to freely suspended cells in bubble-free bioreactors (Papoutsakis, 1991b; Chisti, 2001). This is so as long as the energy input in the fluid is such that the size of the fluid microeddies remains significantly larger than the dimensions of the cells (Chisti, 2001). This condition is nearly always satisfied in cell culture bioreactors. Supplying oxygen through surface aeration is sufficient for culture scale < 5 L, but once the volume exceeds 5 L, other means of supplying oxygen are necessary (Humphrey, 1998). At large scale, the most simple and effective method of aeration is by sparging the culture broth with a gas mixture (Chisti, 2000). However, sparging has been shown to damage animal cells (Butler *et al.*, 1999; Passini and Goochee, 1989; Cherry and Hulle, 1992; Handa-Corrigan *et al.*, 1989; Jöbses *et al.*, 1991). Nevertheless, most large-scale animal cell suspension cultures are aerated by controlled sparging.

Extensive information is available on the effects of shear forces on animal cells in defined laminar flow fields, such as those in viscometers (Mardikar and Niranjan, 2000; Shiragami, 1997; Ramírez and Mutharasan, 1990b; Petersen *et al.*, 1988 and 1990;

Smith *et al.*, 1987) and capillaries (McQueen *et al.*, 1987). The information regarding shear fields in the stirred bioreactor, however, is limited (Chisti, 1999).

Shear rate (γ) is a measure of spatial gradient of velocities between two streams in a fluid (Merchuk, 1991). Shear stress (τ) is sometimes used to report the magnitude of the fluid mechanical force. In Newtonian laminar flow, these two parameters are related by the viscosity of the fluid (μ) as follow:

$$\tau = \mu\gamma$$

In the turbulent environment of a stirred bioreactor, both these quantities are difficult to measure as the shear rate changes with both location and time within a vessel (Chisti, 1999; Merchuk 1991). Nevertheless, many researchers have attempted to characterize either an average shear rate or a maximum shear rate in different types of bioreactors as discussed elsewhere (Chisti, 1999, 2001).

2.4.4.1 Mechanisms of cell damage

Many studies have shown that damage to freely suspended cells is mostly associated with gas bubbles (Kunas and Papoutsaki, 1990a; Gardner *et al.*, 1990; Jordan *et al.*, 1994; Cherry and Hulle, 1992; Honda-Corrigan *et al.*, 1989; Jöbses *et al.*, 1991). In a sparged bioreactor, gas bubbles are formed at the gas sparger located below the impeller. The bubbles then rise through the liquid to the surface. Finally the bubbles reach the liquid surface and burst. Therefore, bioreactor can be divided into three regions where cells go through different level of shear stress. Jöbses *et al.* (1991) reported that both bubble formation and bubble rising regions had no effect on cell death. Bubble break-up at the surface has been identified as the principal cause of damage to suspended cells in sparged bioreactors (Honda-Corrigan *et al.*, 1989; Cherry and Hulle, 1992; Jöbses *et al.*, 1991; Chisti, 2000).

Mechanical agitation without sparging can also drag gas bubbles into the culture medium (Wu, 1995). Butler *et al.* (1999) reported that cell damage occurred at greater than 200 rpm stirring rate in stirred bioreactor, owing to bubble disengagement at gas-liquid interface and bubble entrainment by vortex formation. However, cells survived at agitation rates of greater than 700 rpm in the absence of gas bubbles (Butler *et al.*,

1999). A similar observation was reported by Kunas and Papoutsakis (1990a), where cells could grow at agitation speeds up to 600-700 rpm if both vortex and bubble entrainment were eliminated. A point of note here is that the cells were protected to a certain extent against damage by the presence of either serum (Kunas and Papoutsakis, 1990a; CRL-8018 hybridoma cells) or linoleic acid (Butler *et al.*, 1999; murine hybridoma CC9C10) during experiments by these researchers. The maximum agitation rate that is withstood by cells in the absence of protective agents such as serum is expected to be lower than the above-mentioned stirring speeds.

Generally, large bubbles are less damaging then small bubbles (Chisti, 2000). As quoted by Chisti (2000), bubbles as large as 10 mm in diameter are less damaging as compared to small bubbles with a diameter of 2 mm. A large bubble has a mobile interface and rises faster than small bubbles. Hence a large bubble carries fewer cells attached on its surface to the surface of the broth. This greatly reduces the chance of cells being exposed to lethal events of bubble rupture at the liquid surface. In contrast, small bubbles with rigid interface carry more cells on their surface as they rise slowly and are stable at the liquid surface. In culture of several hybridoma cell lines in commercial stirred tank bioreactors up to 300 L, Chisti (1993) observed no sign of cell damage when submerged aeration with bubbles of 10-20 mm diameter was used. The culture performance was comparable to that in the roller bottles. In contrast, aeration with sintered metal spargers, which produced slower rising bubbles of 2-3 mm in diameter, caused the cells to rapidly rise to the stable foam layer at the liquid surface.

The size of a cell is typically 10 - 20 μm, but the thin film between bubbles at the gas-liquid interface is around 0.2 - 2.0 μm thick (Papoutsakis, 1991b). As a result, cells are trapped not between the films but in the liquid between bubbles (lamellae) (Figure 2.1). When the liquid film drains, this film becomes thinner and the stretching three sides of lamellae, cause stress on the cells trapped within. For bubbles at the liquid surface, when the bubble film becomes sufficiently thin, a hole develops at the center and the film moves back into the bulk liquid rapidly. The fluid accelerates down the interior walls of bubble cavity and this has been shown to impose high stresses on the cells adhering to the bubble cavity (Cherry and Hulle, 1992). The impact of the flows behind the bubble leads to the ejection of an upward fluid jet above the liquid surface, and an equal but opposite jet below the liquid surface. These high speed impacts and

flows kill cells in their vicinity (Papoutsakis, 1991b; Chisti, 2000). Bubbles larger than ~ 6 mm in diameter burst less violently; the speed of the jet, the maximum pressure produced and the maximum energy-dissipation rate decline with increasing bubble diameter (Chisti, 2000).

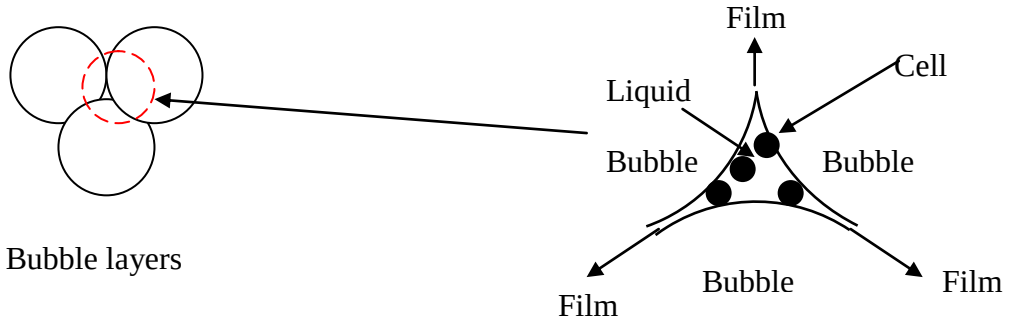


Figure 2.1: Lamellae between bubble layers (adapted from Papoutsakis, 1991b**)**

2.4.4.2 Protective agents

The use of stirred bioreactors is unavoidable in large scale cultivation of mammalian cell, be it suspension or adherent cells immobilized on microcarriers. Provision of sufficient oxygen through sparging is therefore unavoidable. A normal practice is to provide the culture with shear protective agents to counter the damaging effects of agitation and sparging.

The sensitivity of cells to shear stress is dependent on the growth phase, growth rate, culture age and media formulation (Butler *et al.*, 1999). Butler and co-workers (1999) proposed two mechanisms of shear protection: biological and physical routes. The biological route was said to enhance the cells' ability to resist shear by causing metabolic changes that required a relatively long period to take effect. Biological mechanism could also involve interactions between the additive and the cell membrane. Examples of biological protective agents are serum (Michaels *et al.*, 1991; Legazpi *et al.*, 2009), linoleic acid (Butler *et al.*, 1999) and bovine serum albumin (Wu, 1995; Francis, 2010). On the other hand, the physical route of protection involved reducing the level and frequency of shear forces experienced by the cells (Butler *et al.*, 1999; Kunas and Papoutsakis, 1990b; Wu, 1995; Chisti, 2000). Protective agents that act via a nonmetabolic physical mechanism include polyethylene glycol (PEG), Pluronic F68, methylcellulose and carboxymethyl cellulose (Michaels *et al.*, 1991; Murhammer and

Goochee, 1990; Chattopadhyay *et al.*, 1995; Kunas and Papoutsakis, 1990b; Butler *et al.*, 1999; Wu. 1995; Chisti, 2000).

According to Michaels *et al.* (1991), FBS was able to protect cells through both biological and physical mechanisms. Nonetheless, at a serum concentration of lower than 1%, serum lost its ability to protect the cells at high agitation speed and the cell concentration declined rapidly (Kunas and Papoutsakis, 1990b; Ozturk and Palsson, 1991b). Chattopadhyay *et al.* (1995) discovered that Pluronic F68, Methocel E50 and Methocel E4M could protect cells from bubble rupture by preventing cells from adhering to the bubble surface. These additives also lower the liquid-vapor interfacial tension of the culture medium (Chattopadhyay *et al.*, 1995). However, addition of Pluronic F68 in the medium can have adverse effects through reducing yield of cell and making purification of the product difficult (Butler *et al.*, 1999).

The most common protective agent utilized in sparged bioreactors is Pluronic F68. Pluronic F68 is a nonionic block of copolymer of polyoxyethylene-polyoxypropylene with an average molecular weight of 8400 Da (Murhammer and Goochee, 1990). The typical concentration used is in the range of 0.5 and 3.0 kg/m^3 [0.05 – 0.3% (w/v)] (Chisti, 2000). 0.2% (w/v) Pluronic F68 was found to give the best protection without foam formation by Murhammer and Goochee (1990). A concentration of 0.1% (w/v) Pluronic F68 is sufficient to protect cells in a sparged and stirred bioreactor without significant foam formation.

2.4.5 Waste products

In mammalian cell culture, glucose and glutamine are required for biomass synthesis. They are also a major energy source. Main products of glucose and glutamine metabolism are lactate and ammonia. It is believed that, as waste products, their accumulation in the medium will limit cell growth and product formation (Schneider *et al.*, 1996; Genzel *et al.*, 2005).

Generally, lactate production in mammalian cell culture is owing to the metabolism of glucose. When glucose is metabolized inefficiently or overflow

metabolism occurs (i.e. the rate of glycolysis exceeds some critical value), a higher concentration of lactate is produced (Zhou *et al.*, 1997; Gambhir *et al.*, 1999). Lactate is also one of the products of glutaminolysis. Production of lactic acid during mammalian cells growth generally lowers the culture pH because of limited buffering capacity of the medium. This inhibits cell growth. An increase in lactate concentration causes a remarkable increase in specific death rate, mainly because of the increase of osmotic pressure due to a build up of sodium lactate in the medium (Omasa *et al.*, 1992; Ozturk *et al.*, 1992). A similar observation was reported by Glacken *et al.* (1986), where sodium lactate formed by the reaction between sodium bicarbonate in the medium and the lactic acid produced by the cells, significantly inhibited antibody production by hybridoma cells in an isotonic (325 mOsm) solution. Inhibitory effect of lactate on cell growth has been reported at a concentration range of 22 – 55 mM (Legazpi *et al.*, 2005; Macmillan *et al.*, 1987; Ozturk *et al.*, 1992).

Ammonia formation in mammalian cell culture is generally attributed to glutamine catabolism. A second important source is through chemical decomposition of glutamine. Glutamine is not chemically stable in culture media. It tends to decompose chemically to produce ammonia and pyrrolidonecarboxylic acid (Ozturk and Palsson, 1990b; Schneider *et al.*, 1996). Thus, the disappearance of glutamine in the culture cannot be ascribed exclusively to uptake by cells and the measured rate of disappearance must be corrected for chemical degradation to accurately establish cellular uptake.

Chemical degradation of glutamine follows first order kinetics:

$$- d[Gln]/dt = k[Gln]$$

where [Gln] is the glutamine concentration, t is time and k is the first order rate constant (Ozturk and Palsson, 1990b). The rate constant k is influenced by the medium composition, pH and temperature (Ozturk and Palsson, 1990b; Schneider *et al.*, 1996). Thus, the k value must be determined at the temperature and pH of use and in the specific medium of interest (Ozturk and Palsson, 1990b; Schneider *et al.*, 1996).

As quoted by Genzel *et al.* (2005), depending on both the cell line and the medium, cell growth will be severely inhibited at ammonia concentration as low as 1 - 5 mM in the culture medium. Martinelle and Häggström (1993) suggested that one of the

important toxic mechanisms of ammonia/ammonium ion exposure is an increased demand for maintenance energy, caused by the need to keep up ion gradients over the cytoplasmic membrane. They demonstrated that ammonia crosses the cell membrane through simple diffusion, while ammonium ion needs a transport protein to do so. Doyle and Butler (1990) examined the effect of pH on the toxicity of ammonia to a murine hybridoma. They showed that at high pH, a lower concentration of ammonium chloride was able to inhibit cell growth compared to at low pH. Hence, Doyle and Butler (1990) suggested that a decreasing of pH during cultivation may counteract the ammonia accumulation effect, which could be advantageous for maximizing cell growth. Nonetheless, it has to be very clear that the effect of adding ammonia to the medium could be very different from that of ammonia produced within the cells.

The common approach in avoiding accumulation of the waste products in mammalian cell culture is to replace the spent medium with fresh medium. Without a doubt, this strategy increases the production costs, especially if using a serum-containing medium. A few strategies have been proposed, which include substitution of glutamine by its stable derivatives, controlled addition of glutamine and glucose, replacement of glucose by other sugars, and removing of ammonium using ion-exchange resins, non-porous ion-exchange membranes, gas-permeable hydrophobic porous membranes and electrodialysis (Butler and Jenkins, 1989; Schneider *et al.*, 1996).

Dalili *et al.* (1990) found that minimum initial glutamine concentration required for a hybridoma cell line that they studied was 2 mM, below it the specific growth rate and specific antibody production rate were reduced. Yet, Genzel *et al.* (2005) successfully replaced 2 mM glutamine with 10 mM pyruvate in the medium without growth rate reduction for several different adherent commercial cell lines and this greatly reduced the production of ammonia and lactate.

Ljunggren and Häggström (1990, 1994) effectively reduced ammonium ion production in the culture medium of myeloma and hybridoma cells by controlled feeding of glutamine in fed-batch culture. A similar finding was reported by other researchers working on 293 HEK cells (Lee *et al.*, 2003), recombinant murine myeloma cells (Gambhir *et al.*, 1999), PER.C6^{TM} cells (Maranga and Goochee, 2006) and

hybridoma cells (Kurokawa *et al.*, 1994; Li *et al.*, 2005). Different cell lines and culture media were used in these studies and their methodology of feeding the components that were being controlled were not identical. When only glucose was controlled at low concentration, lactate production was reduced, but ammonia accumulation increased (Ljunggren and Häggström, 1994; Kurokawa *et al.*, 1994). On the other hand, if only glutamine was controlled at a low concentration, lactate, alanine and ammonia accumulation were all reduced (Ljunggren and Häggström, 1994; Kurokawa *et al.*, 1994; Lee *et al.*, 2003). Conversely, when both glucose and glutamine were controlled at low levels, accumulation of both lactate and ammonia were almost totally eliminated (Maranga and Goochee, 2006; Kurakawa *et al.*, 1994; Li *et al.*, 2005; Ljunggren and Häggström, 1994; Gambhir *et al.*, 1999). The feeding concentrations for glucose and glutamine of these researchers were 0.28 – 1.10 mM and 0.085 – 0.700 mM, respectively.

Glacken *et al.* (1986) has explored the possibility of substituting glucose with other sugars. Galactose was found to be able to substitute for glucose and reduce the specific lactic acid productivity dramatically. Altamirano *et al.* (2004) developed a fed-batch strategy to cultivate t-PA (tissue type plasminogen activator) producing CHO cells by substituting glutamine with glutamate and alternating glucose with galactose. Lactate consumption in glucose/galactose-fed culture was significantly higher than in glucose-fed culture, which meant that lactate was co-metabolized with galactose. They concluded that the higher cell concentration and viability obtained were mainly the consequence of glucose substitution by galactose, rather than lactate detoxification.

Brose and van Eikeren (1990) developed a membrane-based method to remove toxic ammonia from mammalian cell culture. Their gas-permeable hydrophobic porous membrane was able to selectively remove ammonia to well below 1 mM. BHK cells were grown in the culture medium that was spiked with 14 mM ammonia, Brose and van Eikeren (1990) successfully used the membrane they developed to strip off the ammonia. The growth rate for BHK cells was found to be similar to those of cells grown in the fresh medium.

Control of initial glucose and glutamine concentrations in the culture medium seems to be critical in ensuring the availability of these nutrients for growth and production, and also reducing the formation of waste products that affect growth.

2.4.6 Inducer agent

The MAb production and secretion by some hybridomas can be very low (Martín-López *et al.*, 2007). Thus, to facilitate rapid generation of milligrams of MAb suitable for initial clinical trials, an alternative is to use inducer agents to improve antibody production and secretion. There are several potential inducers, including amino acids, lipopolysaccharides (LPS), dimethyl sulfoxide (DMSO), anti-mouse IgG, lysozyme and interleukin-6.

Supplementation of the culture with amino acids was found to enhance antibody production rate by several researchers (Ducommun *et al.*, 2001; Gong *et al.*, 2006; Hiller *et al.*, 1994). In addition, feeding of amino acids during fed-batch culture has allowed cells to maintain a high viability for a longer period (Ducommun *et al.*, 2001). This is important as more antibody can be produced if an actively producing cell lives longer. Amino acids have also been found to promote cell growth (Franěk *et al.*, 2003; Gong *et al.*, 2006; Qi, 1993) as they are the main nitrogen source in media. Certain essential amino acids, such as threonine, have been found to protect CHO cell from hyperosmolality stress (deZengotita *et al.*, 2002) and ammonium stress (Chen and Harcum, 2005).

LPS is a polyclonal mitogen which is able to stimulate a strong response from the normal animal immune system (Martin-Lopez *et al.*, 2007; Oliver *et al.*, 1999). It has been found to be able to induce the differentiation of cells that are synthesizing IgG and IgA (Kearney and Lawton, 1975; Martin-Lopez *et al.*, 2007; Oliver *et al.*, 1999). Yoshinari *et al.* (1996) found that together with a 1/500 (v/v) dilution of pokeweed mitogen and 100 U/mL of interleukin-4, 20 µg/mL of LPS in culture medium could stimulate the production of human IgG. Martin-Lopez *et al.* (2007) found that when hybridoma cells were co-cultured with LPS and anti-mouse IgG, both cell proliferation

and antibody production rates increased. They suggested that timing of LPS and anti-mouse IgG addition played a critical role in enhancing MAb production.

Although DMSO induces apoptosis, increases membrane fluidity and permeability (Kawai and Nishizawa, 1984; Marthyn *et al.*, 1998), it has been shown to aid in DNA transfection (Kawai and Nishizawa, 1984) and monoclonal antibody production (Ling *et al.*, 2003). Adding 0.2% DMSO at the instance of maximum viable cell density has been shown to increase specific MAb production by about two-fold (Ling *et al.*, 2003). Similarly, 81% increase in hepatitis B surface antigen production and 3-fold increment in specific productivity of CHO cell line was demonstrated by Wang *et al.* (2007) with the addition of 1.5% DMSO at 96 h. Cell growth was depressed, but cell death and lysis were not significant (Wang *et al.*, 2007).

Lysozyme has also been identified as an immunoglobulin production stimulating factor by Murakami *et al.* (1997). Lysozyme concentration at 380 µg/mL was found to enhance IgM production by HB4C5 cells more than 13-fold in a serum-free medium. In addition, lysozyme also increased production of IgM and IgG from human peripheral blood lymphocytes. These immunoglobulin production enhancements were not accompanied by growth promotion. According to Murakami *et al.* (1997), the stimulating activity of lysozyme was not as a result of its enzymatic activity or reaction products.

Other substances have been identified as immunoglobulin production-stimulating factors, e.g. lactate dehydrogenase (Takenouchi and Sugahara, 2003), polyamines (Sugahara *et al.*, 2008), histone (Okamoto *et al.*, 2002), alcohol dehydrogenase-I (Okamoto *et al.*, 2001) and rice fermented seasoning (Sugahara *et al.*, 1996). All of these either stimulated production of IgM or IgG from different cell lines.

In view of the advantages noted above, essential amino acids, LPS, lysozyme and DMSO, were screened in this work for their effectiveness in inducing MAb production of hybridoma 192. They were selected because of their availability, consistency in quality and a relatively low cost as compared to the other substances used by other researchers.

2.5 Scale-up

Large quantities of monoclonal antibody are required in a clinical study as well as in evaluation of a diagnostic assay in a clinical setting. Production in small cultures can not meet this demand as hybridoma cells are typically low producers (Martín-López *et al.*, 2007). Setting up multiples of small cultures is not practical either, as it is tedious, expensive and also labor intensive. Hence, a scale-up of the size of the culture vessel and other systems is a more practical route to production. Unfortunately, bioreactors cannot be scaled up in such a way as to exactly duplicate at different scales all the factors that influence production. Therefore, operational protocols and bioreactor designs may need to be adapted to achieve the best performance at different scales.

2.5.1 Scale up of culture processes

Stirred tank bioreactors are most commonly used to scale up production of suspension culture cells. In part, this is due to the vast information and knowledge available about the design and operation of a stirred tank bioreactor. To some extent, this is attributable to the stirred tank reactor being a common type of bioreactor that was the basis for obtaining most of the FDA approvals in producing of the cell culture derived therapeutic and diagnostic products.

For volume independent parameters such as dissolved oxygen, pH and temperature, values are set at the same level in large scale as on the small scale. While for volume dependent parameters such as total air flow rate and agitation speed, settings used on small scale may be totally inadequate at the larger scale. Problems such as mixing, shear and oxygen limitation may be created by scale changes. Nevertheless, scale up of total air flow rate is relatively simple. This can be done by normalizing the volumetric flow rate with respect to the vessel working volume. The major difficulty in scale up will be more on the agitation speed. Agitation speed of the small scale when utilized in large scale may not be sufficient to provide homogenous mixing and oxygen transfer. However, a higher speed may introduce agitation and aeration associated damage to the culture. Therefore, the new speed should ensure both mixing and oxygen transfer are adequate. As a rule of thumb, industrial scale up is based on geometric

similarity and maintaining equal tip speed, equal mass transfer coefficients, or equal specific energy dissipation rates (Pollard *et al.*, 2007; Yang *et al.*, 2007; Garcia-Ochoa and Gomez, 2009) depending on the situation.

2.5.2 Problems in scaling-up

The major difficulties to date in scaling-up cell culture include oxygen supply limitation, waste product buildup, shear sensitivity of the animal cells and the need for advanced process control (Chu and Robinson, 2001; Nienow, 2006). System geometry, impeller design, fluid properties and operating parameters greatly influence transport phenomena in the bioreactor at different scales (Pollard *et al.*, 2007). Considering the differences in sensitivity of cells to these factors, the selection of scale-up criteria is not straightforward. The selection should be primarily based on the property that is most critical in determining the performance of the process.

Factors that affect the success of scaling up an animal cell culture are highly dependent on whether the physical and chemical requirements of the cells have been fulfilled (Votruba and Sobotka, 1992). Therefore, scale-up studies are required to be carried out in order to identify the conditions and parameters that will give the same process result at a larger scale (Reisman, 1993) as on the small scale. Insufficient attention to scale up was in the past a cause of many failures in commercializing products from animal cell cultures.

2.5.3 Scale up methods

Bioreactors are typically scaled up such that scale change is in the ratio of 1:10 in one stage. Several stages of scale up may be needed (Garcia-Ochoa and Gomez, 2009). As discussed by Garcia-Ochoa and Gomez (2009), four approaches are normally used. The influences of operational conditions and bioreactor geometry on the flow pattern in the bioreactor are described by mathematical models in more fundamental scale up methods. This approach is very complicated and needs a lot of simplification of the model. In semi-fundamental methods, simplified equations are used to obtain a practical approximation to the bioprocess operation, but the method is still complex.

Dimensional analysis is used to keep the values of certain dimensionless groups of parameters constant during scale-up. Nevertheless, it is impossible to keep all dimensionless groups constant and a decision has to be made about the choice of the most important ones. Finally, the most common method is the rule of thumb method that is based on prior experience.

According to Ju and Chase (1992), there are three conventional scale-up strategies. First, the ratio of impeller diameter (D_i) to bioreactor diameter (D_T), the volumetric oxygen transfer coefficient (k_La) and the volumetric gas flow rate (Q) per unit reactor volume are maintained constant in moving from one scale to another. Then, the impeller speed is determined through a k_La correlation. Secondly, the k_La, D_i/D_T and the impeller tip speed (ND_i where N is the rotational speed) are kept constant and Q is calculated from the k_La correlation. In a third strategy, k_La, ND_i and Q are maintained constant, while D_i/D_T is adjusted. The choice is specific to the individual cell line and process, and the most critical factor that affects the performance of the particular process.

2.5.4 Scale up strategy for shear sensitive hybridoma cell line

For a shear sensitive cell line, constant impeller tip speed or shear criteria are normally used. Nevertheless, its use results in reduction of stirrer speed and power input per unit volume on scale up, which in turn reduces oxygen transport rate (Garcia-Ochoa and Gomez, 2009). Since the oxygen consumption rate for mammalian cell cultures is lower than of microbial cell cultures, and the shear sensitivity is higher than for microbial cell, this option will be evaluated in this research.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Cell line and stock preparation

3.1.1 Hybridoma cell and growth conditions

Hybridoma 192 was a gift from the Animal Cell Culture Laboratory, Physiology Department, Medical Faculty, University of Malaya, Malaysia. This cell line was the result of fusion between mouse myeloma cells (P3X63 Ag8.653) and mouse lymphocytes. It was able to produce monoclonal antibody against the steroid hormone 17 alpha-hydroxyprogesterone. The medium used for maintenance of cells was Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemicals, St. Louis, MO, USA) supplemented with 5% v/v Fetal Bovine Serum (FBS) (Gibco, Invitrogen Corporation, USA), 1% v/v antibiotic-antimycotic (Gibco, Invitrogen Corporation) and 4 mM L-glutamine (Sigma). Cells were typically incubated at 37°C in a humidified CO_2 incubator (Shel Lab, USA) with an atmosphere of 5% v/v CO_2 in air.

3.1.2 Thawing and freezing of cells

Cells were thawed by warming the vial in a water bath at 37°C for 40s immediately after removing from the master cell bank (-196°C) or working cell bank (-82°C). Thawed cells were then diluted twenty-fold in the complete medium (i.e. the maintenance medium; Section 3.1.1). Cells were centrifuged at 120*g* (Centrifuge 5810R, Eppendorf, Germany) for 5 min and re-suspended in 10 mL of fresh medium to remove dimethyl sulfoxide (DMSO) (Sigma). Cells were cultivated in T-25 flasks in a humidified CO_2 incubator with a 5% CO_2 at 37°C. After 6 hours, the medium was replaced with 10 mL of fresh medium. Medium replacement was performed again the next day and incubation continued as specified above.

When cells had recovered from the cytotoxicity effect of DMSO, they were grown to a sufficient concentration. When the viability had reached 90% or more, they

were ready for freezing. For freezing, the cells were centrifuged at 120*g* for 5 minutes and re-suspended in DMEM supplemented with 10% FBS and 10% DMSO to a cell density of greater than 15×10^6 cells/mL. Vials containing 1 mL of the cell suspension were placed in a Mr. Frosty (Nalgene, USA) and frozen at -80°C overnight prior to storage in a liquid nitrogen cell bank as stock cultures.

3.1.3 Stock preparation

Following thawing as explained in Section 3.1.2, the cells were diluted every 2 days with fresh medium to attain a cell density of 2×10^5 cells/mL. The cell broth was then split and expanded in T-75 flasks, followed by expansion in a 250 mL spinner flask. The freezing process described in Section 3.1.2 was then used to store stock. For each experiment, one vial was taken from the cell bank and was cultured in the same manner as described in the preparation of cell stock. When the cell viability had reached 90% or higher, they were ready for use in various experiments. Cells were not centrifuged to remove the medium prior to use in inoculation in various experiments, unless otherwise stated. This approach imitated the typical production protocol that is used in most commercial processes for making monoclonal antibodies.

3.2 Preparation of culture media

3.2.1 Basic media preparation

All powdered media were purchased without added sodium bicarbonate ($NaHCO_3$). Supplements were added prior to sterile filtration, or were introduced aseptically to sterile medium, during complete medium preparation. All preparations were done in a laminar flow hood, except preparation of media and supplements that required sterile filtration just prior to use.

Exact amount of the required powdered medium was measured. About 80-90% of final required volume of ultra pure water (UP) at a temperature of between 20° and 30°C was added to an appropriate mixing vessel. Powdered medium was added slowly to the UP water while gently stirring with a magnetic stirrer. Powdered medium was

quantitatively transferred to the mixing vessel. The medium was allowed to mix for at least 30 minutes, as recommended in Sigma's product manual.

Appropriate amount of $NaHCO_3$ (3.7 g/L for DMEM) was added to the medium and allowed to mix for a further 10 minutes. Pluronic F68 (0.1% w/v; Sigma) was then added and mixing was continued for a further 20 minutes. pH of the medium was adjusted to 0.1-0.2 units below the desired final pH (pH 7.4) while mixing. This was done by using 1 N hydrochloric acid (HCl). The volume was brought up to the final desired level by adding UP water. Mixing was continued for at least another 30 minutes. All reagents used were of 'cell culture' grade.

The medium was then sterile filtered immediately by using a membrane filter unit with a porosity of 0.22 µm or less (Supor®machV, PES membrane, Nalgene, USA). Aliquots of the medium were distributed into sterile bottles for easier storage. The basal media were stored at 4°C in the dark until use. The maximum storage period was one month.

3.2.2 Preparation of complete media

Complete media were prepared by adding the filter sterilized liquid supplements according to the formula for each medium as in Table A.1 (Appendix A) into a 500 mL sterile Schott bottle. Basal DMEM was then poured and topped up to the required volume. The bottle was swirled for a few minutes, labeled and ready to be used.

3.3 Analytical methods

Samples taken from bioreactor/spinner flask/T-flask were centrifuged at 500*g* for 10 min (Biofuge pico, Heraeus or Sorvall Evolution RC, Kendro). The supernatant was used for analyses of metabolites and MAb. Cell count was done immediately after a sample had been collected, prior to centrifugation. Cell-free supernatant was preserved at -20°C until analysis. Storage prior to analysis did not exceed 60 days.

3.3.1 Total and viable cell count through Trypan Blue dye exclusion

Total and viable cell numbers were determined by the well-known Trypan Blue dye exclusion method (Patterson, 1979; Freshney, 2005). A hemocytometer was prepared for cell counting by cleaning the surface of the slide with 70% alcohol. A clean coverslip was placed over the counting chamber. A sample of cells culture broth was vortexed for 3 seconds for uniform mixing. Cell suspension (30 μL) was mixed with 30 μL (for 1:1 dilution) of Trypan Blue dye (0.4% w/v Trypan Blue in phosphate buffer saline (PBS); pH 7.0) by vortexing for 3 seconds. This mixture (10 μL) was transferred to the hemocytometer chamber by placing the pipette tip at the edge of the coverslip and allowing the cell suspension to fill the chamber by capillary action. The entire volume of the chamber was filled without overfilling. The slides was left standing for 1-2 min, but not longer, as the viable cells deteriorate and take up stain with longer exposure (Freshney, 2005). The cells were viewed under the microscope (100 × magnification). Dead cells stained blue, while live cells excluded the dye. Total and viable cells were counted in the four 1 mm^2 areas marked "W" in Figure 3.1. Only the cells that were on the top and right-hand lines of each square were counted, not those on the bottom and left-hand lines. This prevented counting the same cell twice. Cells on both upper and lower chambers were counted. If the cell concentration calculated from the count exceeded 10^5 cells/mL, the sample was diluted with PBS to attain ~10^5 cells/mL and re-read. Accurate reading of a hemocytometer requires the cell count to be in the range of $3 - 5 \times 10^5$ cells/mL (Patterson, 1979). For an accurate determination, the total number of cells overlying one 1 mm^2 should be between 15 and 50 (Cascade Biologics, Inc., 2002). Reproducibility was checked by repeating the count with a fresh duplicate sample. The cell concentration N was determined as follows:

$$N = \text{cells (total or viable) counted} \times \tfrac{1}{8} \times \text{dilution factor} \times 10^4 \quad \text{(cells/mL)}$$

The cell viability was calculated as follows:

$$\text{Cell viability} = (\text{viable cells} / \text{total cells}) \times 100 \ (\%)$$

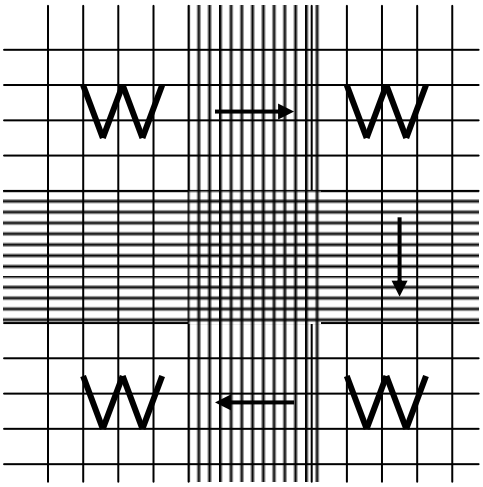


Figure 3.1: Hemocytometer

The hemocytometer slide was cleaned immediately afterwards by washing with 70% isopropanol and air-drying.

3.3.2 Determination of glucose and lactate concentrations for samples from static culture

Glucose and lactate concentrations were determined by using a glucose/lactate analyzer (Pico TRACE, TRACE Analytics Gmbh, Braunschweig, Germany). The device was calibrated before each measurement by using standard solutions of glucose and lactate that were supplied with the instrument. Either a capillary (provided with the device) or a micropipette was used to transfer 10 μL sample into the sample cup. The measured concentrations (g/L) of glucose and lactate were then directly read from the device.

3.3.3 Determination of glutamine and/or amino acids concentrations for samples from static culture

Glutamine and/or amino acid concentrations in the culture supernatant were determined using High Performance Liquid Chromatography (HPLC). Prior to the assay, samples were deproteinated by adding 150 µL trichloroacetic acid (20% w/v, g/100 mL) to 510 µL of the supernatant. This mixture was centrifuged at 1000*g* for 10 minutes using a microcentrifuge (Biofuge pico, Heraeus). Following this, 170 µL $KHCO_3$ (25% w/v) was added to 500 µL of the supernatant above (adapted from Xie, 1997). Samples were then filtered into a microvial and were ready to be analyzed. Amino acids separation kit from Agilent (Agilent 1100 series modules and systems, Agilent Technologies, USA) was used for HPLC. This separation kit employed an automated two-step precolumn derivatization. The online derivatization was performed using ortho-phthalaldehyde (OPA) for the primary amino acids (i.e. aspartic acid, glutamic acid, asparagines, serine, glutamine, histidine, glycine, threonine, alanine, arginine, tyrosine, valine, methionine, norvaline, tryptophan, phenylalanine, isoleucine, leucine and lysine) and 9-fluorenylmethylchloroformate (FMOC) for the secondary amino acids (i.e. hydroxyproline, sarcosine and proline). The column used was Hypersil AA-ODS (5 µm, 200 x 2.1 mm id, Agilent). Solvent A was composed of 2.72 g/L sodium acetate tri-hydrate, 90 µL triethylamine (TEA) and 1.5 mL tetrahydrofuran (THF) at pH 7.2. Solvent B was prepared with 2.72 g/L sodium acetate trihydrate, 40% v/v acetonitrile and 40% v/v methanol at pH 7.2. All chemicals used were of HPLC grade.

The amino acids elution was carried out at a flow rate of 0.45 mL/min. A gradient of mobile phases was used. Elution began with 100% solvent A and reduced slowly to 40% at 17 min. At 18 min, elution solvent was 100% solvent B with a flow rate of 0.45 mL/min. This flow rate was maintained for 0.1 min and then slowly increased to 0.8 mL/min at 18.5 min. With 100% solvent B, this flow rate was maintained until 23.9 min, and then reduced to 0.45 mL/min again at 24 min. With this flow rate, the percentage of solvent A was increased until 100% in 1 min. The column temperature was 40°C throughout.

The detectors used were DAD (diode array detector). Signal A was 338/10 nm with reference wavelength at 390/20 nm. Signal B was set at 262/16 nm with reference wavelength of 324/8 nm. At 15 min, signal A setting was changed to 262/16 nm with reference wavelength of 324/8 nm.

Sample injection was programmed for precolumn derivatization, where 5 μL borate buffer was mixed with 1 μL OPA reagent. The injection needle was automatically washed with water before drawing a 1 μL sample. Mixing was done with maximum speed in air for six times, followed by needle washing with water. One μL of FMOC was then withdrawn and was followed by needle washing with water. These were mixed in air with maximum speed for 3 times. The mixture was then injected into the system. The whole protocol was run automatically for each sample.

In keeping with the manufacturer's recommendations, the derivatization reagents, borate buffer, amino acid standard, and wash water were replaced daily. Similarly, mobile phases A and B were replaced every 2 days with freshly-made solvents. Retention times and response factors were recalibrated daily.

3.3.4 Determination of ammonia concentration for samples from static culture

The concentration of total ammonia in the culture medium was determined via an enzymatic assay kit (Boehringer Mannheim/R-Biopharm, Germany). The assay measured the change in concentration of reduced nicotin-amide-adenine dinucleotide (NADH) when ammonia in the culture supernatant reacted with 2-oxoglutarate to L-glutamate in the presence of glutamate dehydrogenase (GIDH), whereby NADH was oxidized as follows:

$$\text{2-Oxoglutarate} + \text{NADH} + NH_4^+ \xrightarrow{\text{GIDH}} \text{L-glutamate} + NAD^+ + H_2O$$

The amount of NADH oxidized in the above reaction is stochiometric to the amount of ammonia present in the culture medium. NADH was determined by means of its light absorbance at 340 nm using uv-visible spectrophotometer (U-1800, Hitachi, Japan).

Before determination using the assay kit, samples were deproteinated. Thus, 29 μL trichloroacetic acid (20% w/v) was added to 100 μL of the sample supernatant in a

1.5 mL centrifuge tube. The mixture was centrifuged at 1000*g* for 10 min (Biofuge pico, Heraues). Following this, 12 μL of 2M NaOH was added to 100 μL of the supernatant from the above, for neutralization (adapted from Xie, 1997). For optimal determinations, samples were diluted to 0.01 – 0.08 g/L and were at pH range of 7 – 8 as recommended by the manufacturer of the assay kit. The amounts of various solutions added to a measurement cuvette are presented in Table 3.1, as recommended by the manufacturer of the assay kit.

Table 3.1: The amount of assay solution mixed for the determination of ammonia concentrations

Reagent	**Volume (mL)**
Reaction mixture (NADH tablets in 2-oxoglutarate and triethanolamine buffer)	1.00
Diluted sample	0.10
Ultra-pure water	1.90
Mix and incubate at room temperature for about 5 minutes. Read absorbance at 340 nm (A_1). Start reaction by addition of:	
Glutamate dehydrogenase solution	0.02
Mix and incubate at room temperature for about 20 minutes. Read absorbance at 340 nm (A_2).	

The concentration of ammonia (Amm) in the culture medium was calculated as follows:

$$Amm = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times [(A_1 - A_2)_{sample} - (A_1 - A_2)_{blank}] \times dilution \times 1.01$$

where A_1 is the absorbance before enzyme (glutamate dehydrogenase) addition, A_2 is the absorbance after enzyme addition, V is the final volume of solution mixture in the cuvette (i.e. 3.02 mL), MW is the molecular weight of ammonia (i.e. 17.03 g/mol), ε is the extinction coefficient of NADH at 340 nm (i.e. 6.3 L/mmol.cm), d is the length of the light path (i.e. 1 cm) and v (mL) is the sample volume.

3.3.5 Determination of glucose, lactate, glutamine and ammonia concentrations for samples from stirred culture

The concentrations of all metabolites in stirred culture (spinner flask, 2 L bioreactor and 20 L bioreactor) were determined using Biochemical Analyzer (YSI 7100, Yellow Springs Instrument, USA). The device was programmed to calibrate automatically every morning and every eight samples tested. Minimum sample volume of 550 μL was required to run with the auto-sampler. For sample volume less than the mentioned amount, manual feeding was used. A total of 75 μL sample was required for a complete test (25 μL for ammonia, 25 μL for glucose and lactate, and 25 μL for glutamine). The measured concentrations (mmol/L) of glucose, lactate, glutamine and ammonia were then directly read from the device. All samples were tested twice and the average concentrations were reported.

3.3.6 Determination of monoclonal antibody concentration

The concentration of total monoclonal antibody was determined using an Enzyme Linked Immunosorbent Assay (ELISA). First, 100 μL (1 μg/mL) of primary antibody (anti-mouse IgG from goat, γ-chain specific, Sigma, M1397) solution that had been prepared in 0.1 M coating buffer (21.2 g/L Na_2CO_3 and 16.8 g/L $NaHCO_3$, pH 9.6) was pipetted using a multi-channel pipette into all 96 wells of the microplate (maxisorp, NUNC). The plate was then incubated at 37°C while being shaken at 100 rpm for 2 h. The wells were washed three times with washing buffer (0.9% NaCl and 0.05% Tween 20) and once with UP water manually, and tapped dry with several layers of paper towel. This was followed by blocking the antibody-coated wells with 200 μL per well of blocking buffer (1% w/v BSA and 0.1% Tween 20). Again, the microplate was incubated at 4°C overnight. After incubation, the wells were washed with the washing buffer and dried as described above. The pre-coated plates were either kept (not more than 2 weeks) in the humidified 4°C refrigerator for future analysis, or processed immediately as follows:

One hundred μL of serially diluted (1:2000, 1:4000, 1:8000, 1:16000, 1:32000, 1:64000, 1:128000) standard IgG (mouse IgG, Sigma, I 5381) and diluted culture

supernatant (sample) were added into wells, and incubated at 37°C while shaking at 100 rpm for 1 ½ h. The washing procedure as described above was then repeated. One hundred μL of diluted secondary antibody (1:1000; anti-mouse IgG peroxidase from goat, γ-chain specific, Sigma, A3673) was added to each well and the plate was incubated for 1 ½ h at 32°C while shaking at 100 rpm. The washing procedure was repeated.

One ABTS tablet (50 mg/tablet, 1112422, Roche Diagnostic) was completely dissolved in 50 mL substrate buffer (16.7 g/L of ABTS buffer, 1112597, Roche Diagnostic). One hundred μL of this substrate solution was then added to each well on the plate and the plate was left to stand for 25 min at 35°C on an orbital shaker (250 rpm) for optimum color development. The plate was then read at 405 nm using a microplate reader (Columbus, Tecan, Austria). The above method was adapted from Chong (2006) and was further optimized.

3.3.7 SDS-PAGE gel electrophoresis of purified protein

The separating and stacking gels were prepared from the stock and working solutions as shown in Table A.2 and Table A.3 in Appendix A. The vertical slab gel unit from Cleaver Scientific (Warwickshire, UK) was cleaned and assembled. The separating gel was prepared by mixing the solutions as indicated in Table A.4 (Appendix A). About 5.8 mL of the solution was poured into each side of the casting apparatus and allowed to polymerize at room temperature. The stacking gel was prepared once the separating gel had polymerized. Stacking gel (1.8 mL) was then poured onto the top of the separating gel at each side. A comb was inserted to make wells for sample loading. The stacking gel was then allowed to polymerize at room temperature. After polymerization, the comb was slowly pulled out from the gel.

Samples were dissolved in either reducing or non-reducing sample buffer (refer Table A.3) in a ratio of 4:1 or greater (depending on their concentration) on the basis of their volume. Molecular weight marker (Precision plus protein Kaleidoscope standards, BioRad) was used directly. Reducing samples were heated for 5 min at 98°C and fast spun for 1 second prior to electrophoresis. All samples were then loaded into the wells

in the volume of 20 µL/well. The slab gel prepared earlier was set into the electrophoresis system which contained electrophoresis buffer preceding the sample loading. Electrophoresis was conducted at a voltage of 150 V for about 60 min.

Upon completion of the electrophoresis, gels were transferred to a container containing 200 mL of ethanol: acetic acid: ultra pure (UP) water (40%:10%:50%) solution (refer Table A.5) for fixation. The gels were agitated gently using a belly dancer (Belly Dancer, Stovall, USA) for 1 h. The gels were then washed with UP water for 3 times at least for a total of 30 min or incubated overnight with several changes of water (changed once after 30 min incubation and left overnight; changed again the next morning and left to incubate for at least 30 min before the next step). After washing, the gel was sensitized with 0.02% sodium thiosulfate (refer Table A.5) for 1 min with gentle and constant agitation as above. Again, the gel was washed and soaked for 20 seconds with gentle agitation. The washing and soaking step was repeated twice. Next, the gel was stained for 20 min in cold (4°C) 0.1% silver nitrate solution (refer Table A.5) with gentle agitation. After that, the gel was washed again 3 times in UP water for 20 seconds each time. The gel was then transferred to another clean container and soaked in UP water with gentle agitation for 1 min. Following this, the gel was developed with gentle agitation using 3% sodium carbonate (refer Table A.5). After sufficient staining, the development was terminated by washing the gel in UP water for 20 seconds and followed by a 5% acetic acid wash for 5 min. The gel was then photographed by using the bio-imaging system from Alpha Innotech (AlphaEaseFC Imaging System). The silver staining protocol above was taken from technical resources website www.pick-n-post.com and Mortz *et al.* (2001).

3.4 Processing and Purification of MAb

For a small culture volume with a high protein content (e.g. sample from DMEM + 5% FBS and DMEM + 2% FBS), processing and purification of MAb were done through conventional and small scale processes, e.g. ammonium sulfate precipitation, dialysis and protein G purification using a mini column. On the other hand, if the culture supernatant had a low protein content and small volume (e.g. sample from LSD in T-flask and spinner flask), ammonium sulfate precipitation and dialysis

were replaced by protein concentration using Vivaspin (Sartorius Stedim, Germany). If the volume was greater than 200 mL and low in protein content (e.g. sample from LSD in bioreactors), pilot scale equipment was used, e.g. cross flow filtration system. The relevant procedures are outlined in Sections 3.4.1 – 3.4.4.

3.4.1 Protein concentration through ammonium sulfate precipitation

Culture supernatant was centrifuged (500*g*) at 4°C for 10 min. The supernatant was then transferred into a beaker placed in an ice bucket and the volume was determined. All of the following steps were performed on ice or at 4°C. An equal volume of the saturated ammonium sulfate solution was added dropwise into the beaker, while the antibody solution was being stirred gently. The mixture was then gently stirred for another 1 h. Subsequently, the mixture was centrifuged (4°C) for 10 min at 1100*g*. The supernatant was carefully discarded and the pellet was re-suspended in 1 mL dialysis buffer (0.02 M sodium phosphate at pH 7). Next, the solution was transferred into a dialysis tubing (10-20 mm in diameter, MWCO = 50 kDa). This solution was then dialyzed against a minimum of 3 changes of 1 L dialysis buffer (composition as above) over 24 h. After that, the solution was centrifuged (4°C) at 1100*g* for 10 min. The supernatant was aliquot into eppendorf tubes and stored at -20°C until further purification. The above procedure was adapted from Chong (2006) and the product profile for Protein G Agarose gel (Roche Diagnostic, Mannheim, Germany).

3.4.2 Protein concentration using Vivaspin

Culture supernatant was centrifuged at 500*g* for 10 min at 4°C. The supernatant was subsequently filtered through a 0.22 µm Nylon syringe filter. The filtrate was then loaded into the 20 mL size vivaspin at a maximum volume of 14 mL per tube (nominal molecular weight cutoff [MWCO] of 50 kDa). The tube was spun at 8000*g* for 22 min at 4°C. The retentate was collected, pooled and aliquot into eppendorf tubes. The tubes were stored at -20°C until further purification.

3.4.3 Protein concentration using cross flow filtration

Prior to filtration through the cross flow unit, culture supernatant was centrifuged (500*g*, 10 min, 4°C) (Sorvall Evolution, Kendro). Subsequently, the supernatant was filtered using a vacuum filtration unit with a 0.45 µm Nylon membrane. The filtrate was then loaded into the reservoir of the cross flow filtration unit (Kvick Lab, GE Healthcare). Filter cassette with a MWCO of 30 kDa and 0.11 m^2 area was used to concentrate the MAb. Supernatant was filtered at a setting of 150 rpm on the cross flow pump. Retentate was channeled back to the reservoir in order to concentrate the protein. When the volume of the supernatant in the reservoir was about ¼ of the starting volume, the permeate valve was closed and the cross flow rate was reduced to 50 rpm (pump setting). The fluid was circulated for about 15 min. Then, the circulation pump was stopped and the product was drained from the system by gravity.

3.4.4 Purification through Protein G column

Protein G column (mini column, BioRad) was packed with 1 mL Protein G Agarose gel (Roche, cat no 1719416) or commercially available HiTrap Protein G column (GE Healthcare; www.gelifesciences.com) was used. The column was equilibrated with 2-5 bed volumes of starting buffer (0.02 M sodium phosphate, pH 7, 0.45µm filtered) each time before sample loading. The pH of the crude antibody (dialyzed sample from ammonium sulfate precipitation or sample from vivaspin concentration) was adjusted to 7 by either addition of starting buffer (for small sample volume) or 1 M sodium phosphate (pH 9 in the case of large sample volume). After that, the sample was loaded and allowed to pass slowly through the protein G affinity column. Next, the column was washed with about 12 mL of starting buffer as above. The bound antibody was then eluted with the elution buffer (100 mM glycine, pH 2.7). The eluate was collected in appropriate fractions (e.g. 1 mL) in a tube containing 80 µL of neutralizing buffer (0.1 M Tris-HCl, pH 9). The fraction tubes were gently mixed with a vortex mixer and kept on ice. Afterwards, the fractions that contained IgG were identified by measuring the OD using a quartz cuvette and a UV-Vis spectrophotometer (Varian, Carry 50) at 280 nm. The concentration of antibody was estimated, where 1 $A_{280\,nm}$ unit = approximately 0.8 mg/mL. Subsequently, these fractions were pooled and

further processed by dialyzing against 3 changes of 1 L of PBS (pH 7) over 24 h or desalting using commercially available HiTrap Desalting column (Amersham Biosciences). The Protein G column was then washed with 10 mL of starting buffer to regenerate the column for the next purification of crude sample or 10 mL of 20% ethanol for storage purposes at 4°C. The above protocols were adapted from Chong (2006) and product profile of Protein G Agarose gel (Roche Diagnostic, Mannheim, Germany).

3.5 Batch Culture Growth and Production Studies

3.5.1 Static flask cultures

To obtain growth and MAb production characteristics of cells grown in static culture, duplicate tissue culture flasks were inoculated at 2×10^5 cells/mL in a particular medium. These cultures were incubated for 4 – 5 days at 37°C with 5% CO_2 without media replacement. Samples were taken twice daily after the first 24 h of culture until the viability dropped to below 30%.

3.5.2 Spinner flask cultures

To obtain growth and MAb production characteristics of cells in spinner flasks, cells were inoculated at 2×10^5 cells/mL in duplicate and incubated at 37°C with 5% CO_2 and stirred at 40 or 75 rpm. The total volume of culture medium was 90 mL in a 100 mL or 250 mL sized spinner flask. Samples were withdrawn from the spinner flask twice daily after the first 24 h of culture until the viability dropped to below 30%. The geometric details of the spinner flask are provided in Appendix B (Figure B1, Figure B2).

3.5.3 Bioreactor cultures

Cells were thawed in T-flask and subcultured into the spinner flask. When the cell density was about 1×10^6 cells/mL and the viability was greater than 90%, they

were used to inoculate the bioreactor. The 2 L bioreactor (Biotron, Korea) was run with 1.25 L working volume of culture medium. It was inoculated at 2×10^5 cells/mL at 37°C. Dissolved oxygen was maintained at 60% of air saturation by sparging oxygen into the culture through a single hole sparger at a rate of 0.5 L/min and controlled by the automatic gas controller. pH was controlled at 7.4 by the use of CO_2 and 7.5% $NaHCO_3$. Stirring speed was maintained at 75 rpm using a marine impeller (5.5 cm in diameter). 20 mL samples were taken 2 - 3 times a day. The full geometric details of the bioreactor, its various systems and the experimental set up are provided in Appendix B (Figures B3 – B6).

3.6 Bioactivity Assay

3.6.1 Identification of suitable antibody concentration

The concentration of purified monoclonal antibody, which was pooled and dialyzed (or desalted) after protein G purification, was determined using ELISA. First, 100 μL (1 μg/mL) of 17α-hydroxyprogesterone conjugated with bovine serum albumin (17OHP-BSA, Sigma) solution that had been prepared in 0.1 M coating buffer (21.2 g/L Na_2CO_3 and 16.8 g/L $NaHCO_3$, pH 9.6) was pipetted using a multi-channel pipette into all 96 wells of the microplate (maxisorp, NUNC). The plate was then incubated at 4°C overnight. The wells were washed three times with the washing buffer (0.9% NaCl and 0.05% Tween 20) and once with UP water manually. The plate was then tapped dry on several layers of paper towel. This was followed by blocking the antibody-coated wells with 200 μL per well of a blocking buffer (1% w/v BSA and 0.1% Tween 20). Again, the microplate was incubated at 37°C for 1½ h while shaking at 100 rpm. After incubation, the wells were washed and dried as described above. The pre-coated plates were either kept (not more than 2 weeks) in the humidified 4°C refrigerator for future use, or used immediately as explained next.

Purified MAb was serially diluted with PBS. One hundred μL of the diluted MAb were added to the assigned well, and incubated at 37°C for 1 h while shaking at 100 rpm. The washing procedure as described above was then repeated. One hundred μL of the diluted secondary antibody (1:1000; anti-mouse IgG peroxidase from goat, γ-

chain specific, Sigma, A3673) was added to each well and the plate was incubated for another 1 h at 32°C. The washing procedure was repeated. One ABTS tablet (50 mg 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid per tablet, 1112422, Roche Diagnostic) was completely dissolved in 50 mL substrate buffer (16.7 g/L of ABTS buffer, 1112597, Roche Diagnostic). One hundred µL of this substrate solution was then added to each well on the plate and the plate was left to stand for 25 min at 35°C on an orbital shaker (250 rpm) for optimum color development. The plate was then read at 405 nm using a microplate reader (Columbus, Tecan, Austria).

A plot of optical density (OD) versus 1 / dilution factor of the purified MAb was plotted. The dilution factor which gave 80% of the saturation OD was identified. This dilution factor was used for specificity (Section 3.6.2) and cross-reactivity tests (Section 3.6.3). The above method was adapted from Chong (2006).

3.6.2 Specificity test

17α-hydroxyprogesterone (17OHP) concentrations of 0, 25, 50, 100, 125, 250, 500, 1000, 1500 and 2000 ng/mL were prepared in PBS from stock solution of 17OHP (1 mg/mL in methanol). Fifty µL of each of these solutions were then added into the assigned pre-coated wells of the microplate (Section 3.6.1). After the addition of 50 µL of diluted MAb (according to the result from Section 3.6.1), the plate was incubated for 2 h, 37°C, while being shaken at 100 rpm. The plate was washed (×3) as in Section 3.6.1 with the washing buffer. The plate was then washed once with UP water and tapped dry as described above.

Approximately 100 µL of diluted secondary anti-mouse IgG conjugated to peroxidase (as in Section 3.6.1) were added into each well and re-incubated at 32°C while shaking at 100 rpm for 1 h. The plate was washed and dried as before. One hundred µL of ABTS solution prepared as in Section 3.6.1 were added into each well and the plate was left to stand at 35°C while being shaken at 250 rpm for 25 min. The absorbance was then read at 405 nm. The above method was adapted from Chong (2006).

3.6.3 Cross-reactivity test

Steroids (e.g. β-estradiol, 17α-hydroxypregnenolone, 5-pregnen-3β-ol-20-one, 4-androstene-3, 17-dione, dehydroisoandrosterone, etc.) concentrations of between 0 – 2000 ng/mL were prepared in PBS as in Section 3.6.2. Fifty µL of each of these solutions were then added into the assigned pre-coated wells of the microplate. Next, 50 µL of the diluted MAb (according to the result from Section 3.6.1) was added into all wells. The plate was incubated for 2 h, 37°C, while shaking at 100 rpm. The plate was washed four times as before and tapped dry. Secondary anti-mouse IgG conjugated to peroxidase (as in Section 3.6.1) at 100 µL volume was added into each well and incubated at 32°C with shaking at 100 rpm for 1 h. The plate was washed and dried as before. ABTS solution that had been prepared as in Section 3.6.1 was added at 100 µL/well into each well. The plate was incubated at 35°C with shaking at 250 rpm. The absorbance was read at 405 nm after 25 min. Possible cross-reactivity could be determined by plotting OD versus steroid concentrations, together with OD versus free 17OHP concentration. The above method was adapted from Chong (2006).

CHAPTER 4:

RESULTS AND DISCUSSION

4.1 Serum Reduction and Baseline Data Collection

4.1.1 Introduction

There are basically three methods of adapting cells to serum-free media: direct adaptation, sequential adaptation or weaning, and adaptation in a conditioned medium. In direct adaptation, cells maintained in a serum-containing medium are inoculated directly into a serum-free medium (Chua *et al.*, 1994; Voigt and Zintl, 1999; Hayavi and Halbert, 2005; van der Valk *et al.*, 2010). Cells are subcultured when the density reaches about 1 - 3 $\times$ 10^6 cells/mL at 90% viability. When the cells density attained is 2 – 4 $\times$ 10^6 cells/mL after 4 to 7 days in culture, the cells are considered fully adapted to serum-free environment.

Sequential adaptation or weaning method is based on gradual elimination of serum (Shacter, 1989; Hesse and Wagner, 2000; Legazpi *et al.*, 2005; van der Valk *et al.*, 2010). In the first passage, cells are cultured in a serum-containing medium. Subsequently, the cells are subcultured in the fresh medium containing progressively reduced serum concentration until the medium is serum-free. Three, four or more sub-culturing stages with progressively reduced serum level are used. This method is commonly used, as it does not expose cells to a sudden stress.

Method of adaptation with conditioned medium (i.e. a medium that has been used to grow cells for one full passage) is similar in principle to the weaning method (Spens and Häggström, 2005; van der Valk *et al.*, 2010) described earlier. The difference is that instead of progressively reducing the concentration of serum, the concentration of conditioned or spent medium is reduced. Conditioned media are believed to provide cells with stimulatory factors.

All three methods discussed above require that the cells used in each inoculation are from the mid-exponential growth phase and have a viability of 90% or more. This

increases the chances of proliferation of the cells. The inoculum cell density is typically kept at greater than the optimal cell density for inoculating the serum-containing medium (Voigt and Zintl, 1999). This increases the likelihood of enough cells surviving in the new environment for growth and subsequent passage.

Obviously a given cell line responds differently to different basal media (Renard *et al.*, 1988; Chua *et al.*, 1994); however, common basal media such as DMEM, have been shown to be broadly useful for cultivating hybridomas. A commonly used (Murakami *et al.*, 1982; Hiller *et al.*, 1994; deZengotita *et al.*, 1998; Lin *et al.*, 1999; Kunkel *et al.*, 2000) or recommended (Barnes and Sato, 1980; Maurer, 1986; Freshney, 1994) basal medium for hybridoma cell lines is DMEM/F12 in the ratio of 1:1 (DF). DMEM is high in nutrient concentrations, whereas Ham's F12 is rich in trace elements and contains more vitamins. Media such as eRDF (i.e. enhanced RPMI: DMEM: F12 in 2:1:1) have been also shown to perform well in cultivating hybridomas (Murakami *et al.*, 1997; Takenouchi and Sugahara, 2003; Inoue *et al.*, 2000a, 2000b; Chua *et al.*, 1994; Shinmoto *et al.*, 1991; Nagira *et al.*, 1995).

This work focused on some of the commonly available, inexpensive, defined and nonproprietary basal media for cultivating the hybridoma 192. Proprietary media compositions were not used. The basal media screening in this study focussed on DF, eRDF and DMEM. The latter had been used to routinely culture the hybridoma 192 cell lines in earlier laboratory studies. The basal media were evaluated based on the cell proliferation, viability and monoclonal antibody productivity. In this study, direct adaptation approach was employed so that a suitable basal medium could be identified rapidly. A basal medium that provided the same volumetric productivity and MAb productivity as did the already existing serum-containing formulation, and a lower expense than the existing medium, was considered satisfactory for further study.

4.1.2 Objectives of study

The objective of the study was to reduce the cells' dependency on serum to the extent possible in order to trim down the cost of production and simplify downstream

processing. Screening of basal media was done in parallel with the intention of improving cell growth and productivity.

4.1.3 Materials and methods

4.1.3.1 Materials

All media and chemicals were from Sigma-Aldrich unless otherwise specified.

4.1.3.2 Serum reduction

Hybridoma 192 cell line was maintained in DMEM supplemented with 5% FBS (Gibco, Invitrogen Corp.), 4 mM L-glutamine and 1% antibiotic-antimycotic (Gibco, Invitrogen Corp). Once the viability was greater than 90%, a cell density of $2.5 – 3.5 \times 10^5$ cells/mL was inoculated into DMEM supplemented with 4 mM L-glutamine and 1% antibiotic-antimycotic using the direct adaptation method. In sequential adaptation, cells were inoculated into the same medium, but with the addition of 3% FBS. Cell growth was then monitored daily through visual inspection under a microscope. Cell count was performed every 48 to 72 hours and the cells were subcultured back to the original cell density. Serum content was further reduced to 1% when the cell morphology was back to normal (i.e. the cell were uniformly round with a smooth surface that was free of any spikes; the cell count increased daily and achieved a density of close to 1 million cells/mL after 48 h) or the cells had adapted to the new environment and cell viability achieved was greater than 90%. Cells were either cultured in DMEM, DF or eRDF (enhanced with 2% MEM amino acids). All media were supplemented with 4 mM L-glutamine and 1% antibiotic-antimycotic. Inoculation density was $2.5 – 3.5 \times 10^5$ cells/mL in all cases. Cells were frozen and kept as stock using respective medium once the viability achieved was greater than 90%. This was done as described in Section 3.1.2. The serum content was then further reduced to 0.5% for DF and eRDF media and the stocks were kept once the cells had adapted to the new environment.

4.1.3.3 Glutamine degradation

Complete DMEM media with 5% serum and 2% serum (pH after filtration was 7.70) were prepared. A 80 mL sample of the freshly prepared complete medium was divided into two 50 mL centrifuge tubes. One tube was placed in the refrigerator (4°C) and the other was held at 37°C in a CO_2 incubator. A 1 mL sample was taken aseptically daily for the first 5 days, then every 2 – 3 days for two weeks, followed by every 7 days for a total of 2 months. All samples were then analyzed using Biochemical Analyzer (YSI 7100, Yellow Springs Instrument, USA) as described in Section 3.3.5.

4.1.3.4 Baseline data collection in T-flask, spinner flask and bioreactor

Adapted cells were cultured as described in Section 3.5. In T-flask batch culture, T-25 flasks were used with a total volume of 10 mL, and 0.8 mL samples were withdrawn each time. For batch culture in spinner flasks, 250 mL spinner flasks were used with a stirring rate of 75 rpm and a total volume of 90 mL. A 2 mL sample was taken from a spinner flask each time. Data presented are the average of six different runs for T-flasks (four for control) and spinner flask cultures, while for the bioreactor the data are an average of 3 separate runs.

4.1.3.5 Fed-batch study in spinner flask

Cells were thawed as usual into T75 flask and subcultured twice before inoculating into 250 mL spinner flasks. When cell density had reached a sufficient value and cell viability was greater than 90%, 2×10^5 cells/mL were inoculated into 2% DMEM. The culture volume was 90 mL. Control culture was also run at different times. All experiments were repeated 3 times. All cultures were kept in humidified CO_2 incubator with 5% CO_2 at 37ºC and an agitation speed of 40 rpm. A 2.0 mL sample was taken immediately after inoculation and twice a day after 24 h. When the viable cell density exceeded 1×10^6 cells/mL, fresh medium was fed up to 230 mL irrespective of the final viable cell density achieved. A sample was taken right after medium feeding and twice a day until the cell viability had declined or the cells entered death phase.

4.1.3.6 Estimation of growth parameters

The parameters determined in this study included the cell growth rate, MAb production rate, glucose consumption rate, lactate production rate, glutamine consumption rate and ammonia production rate. These were used to establish whether the metabolism of hybridoma 192 was affected by serum weaning and cultivation in different modes. The equations used for determination of the parameters are outlined below:

1. Specific growth rate (μ), 1/h

$$\frac{dX_v}{dt} = \mu \cdot X_v \qquad \text{(Eq. 4.1.1)}$$

Where X_v is viable cell density and μ is the average specific growth rate. μ was determined during exponential growth phase through plotting a graph of $ln\ (X_v/X_{vo})$ versus t and fitting a straight line through the origin. At origin, $ln\ (X_v/X_{vo})$ is zero, but the X_{vo} is the value at which the $ln\ (X_v/X_{vo})$ versus t line first becomes straight. The slope of this straight line is μ (Stoll *et al.*, 1996).

2. Specific MAb production rate (r), pg/cell.h

$$\frac{dMAb}{dt} = r \cdot X_v \qquad \text{(Eq. 4.1.2)}$$

Where *MAb* is the concentration of monoclonal antibody and *r* is the average specific MAb production rate. Total MAb produced was calculated by taking into consideration the volume correction for each sampling point up to the maximum value. *r* was then calculated by dividing the total MAb produced with the viability index ($\int X_v\ dt$) at the maximum point (Xie and Wang, 2006).

3. Specific glucose consumption rate (qGLU), mmol/10^9 cells.h

$$\frac{dGLU}{dt} = -qGLU \cdot X_v \qquad \text{(Eq. 4.1.3)}$$

Where *GLU* is the concentration of glucose and *qGLU* is the average specific glucose consumption rate. Total glucose consumed was calculated by taking into consideration the volume correction for each sampling point up to the minimum value. *qGLU* was then calculated by dividing the total glucose consumed with the viability index ($\int X_v\ dt$) at the minimum point (Xie and Wang, 2006).

4. Specific lactate production rate (qLAC), mmol/10^9 cells.h

$$\frac{dLAC}{dt} = qLAC \cdot X_v \qquad \text{(Eq. 4.1.4)}$$

Where *LAC* is the concentration of lactate and *qLAC* is the average specific lactate production rate. Total lactate produced was calculated by taking into consideration the volume correction for each sampling point up to the maximum value. *qLAC* was then calculated by dividing the total lactate produced with the viability index ($\int X_v\, dt$) at the maximum point (Xie and Wang, 2006).

5. Specific glutamine consumption rate (qGLN), mmol/10^9 cells.h

$$\frac{dGLN}{dt} = -qGLN \cdot X_v \qquad \text{(Eq. 4.1.5)}$$

Where *GLN* is the concentration of glutamine and *qGLN* is the average specific glutamine consumption rate. Total glutamine consumed was calculated by taking into consideration the volume correction for each sampling point up to the minimum value. *qGLN* was then calculated by dividing the total glutamine consumed with the viability index ($\int X_v\, dt$) at the minimum point (Xie and Wang, 2006).

6. Specific ammonia production rate (qAMM), mmol/10^9 cells.h

$$\frac{dAMM}{dt} = qAMM \cdot X_v \qquad \text{(Eq. 4.1.6)}$$

Where *AMM* is the concentration of ammonia and *qAMM* is the average specific ammonia production rate. Total AMM produced was calculated by taking into consideration the volume correction for each sampling point up to the maximum value. *qAMM* was then calculated by dividing the total ammonia produced with the viability index ($\int X_v\, dt$) at the maximum point (Xie and Wang, 2006).

7. Lactate yield coefficient on glucose ($Y_{LAC/GLU}$, mmolcell^{-1}h^{-1}/ mmolcell^{-1}h^{-1}) (Xie and Wang, 2006)

$$Y_{LAC/GLU} = \frac{qLAC}{qGLU} \qquad \text{(Eq. 4.1.7)}$$

8. Ammonia yield coefficient on glutamine ($Y_{AMM/GLN}$, mmolcell^{-1}h^{-1}/mmolcell^{-1}h^{-1}) (Xie and Wang, 2006)

$$Y_{AMM/GLN} = \frac{qAMM}{qGLN} \qquad \text{(Eq. 4.1.8)}$$

4.1.4 Results and discussion

4.1.4.1 Serum reduction

Serum reduction using the direct adaptation method was not successful. This method seemed to be too harsh to this cell line as all cells died after 48 h of culture (data not shown). Hence, the sequential adaptation method was used.

Hybridoma 192 cell line could be adapted to DMEM containing 3% FBS after 3 passages. Figure 4.1.1 shows the viable cell density attained after 48 h of culture on subsequent serum reduction to 1% in different media after 3 passages. Hybridoma 192 cell line was not able to survive in DMEM supplemented with 1% FBS if extra supplements were not provided. Nonetheless, cells could grow up to 0.95×10^6 cells/mL and 1.2×10^6 cells/mL after 48 h of culture in DF and eRDF, respectively.

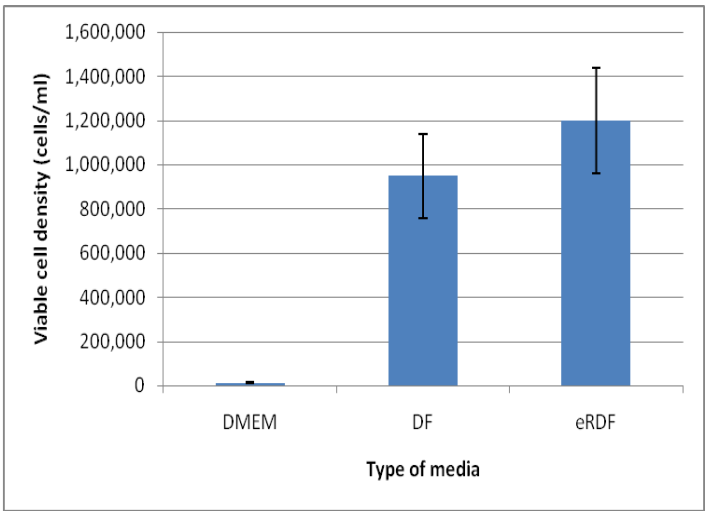


Figure 4.1.1: Viable cell density after 48 h and 3 passages in different basal media supplemented with 1% FBS

Following this, serum content was further reduced to 0.5% in DF and eRDF, and 2% in DMEM. After 3 passages, all cells had adapted to the respective media. Figure 4.1.2 illustrates the specific growth rates, the maximum MAb titer and maximum viable cell density attained for cells that were adapted to these low serum media in comparison with the control (DMEM supplemented with 5% FBS). These figures show that even though cells could grow in the respective media, the growth rates and productivities were significantly different in media which had only 0.5% serum. Figure 4.1.2A shows

that specific growth rate for cells in DF was the lowest (0.021/h) as compared to control (0.032/h). This was followed by cells in eRDF (0.026/h). Specific growth rate for cells in DMEM with 2% FBS (0.034/h) was comparable to control. Similarly, maximum viable cell density attained in DMEM with 2% FBS was as good as in the control culture (1.2×10^6 cells/mL versus 1.38×10^6 cells/mL, as shown in Figure 4.1.2B). However, maximum viable cell density that could be achieved in eRDF and DF supplemented with 0.5% FBS was rather low, at 0.9×10^6 cells/mL and 0.55×10^6 cells/mL, respectively. Figure 4.1.2C shows the maximum MAb titer that was achieved. The MAb titer of cells in DF and eRDF supplemented with 0.5% FBS was extremely low. MAb production of cells in DMEM supplemented with 2% FBS was also low (0.477 µg/mL versus 0.700 µg/mL in control), and the variation between repeated runs was large. Further reduction of serum was stopped at this stage.

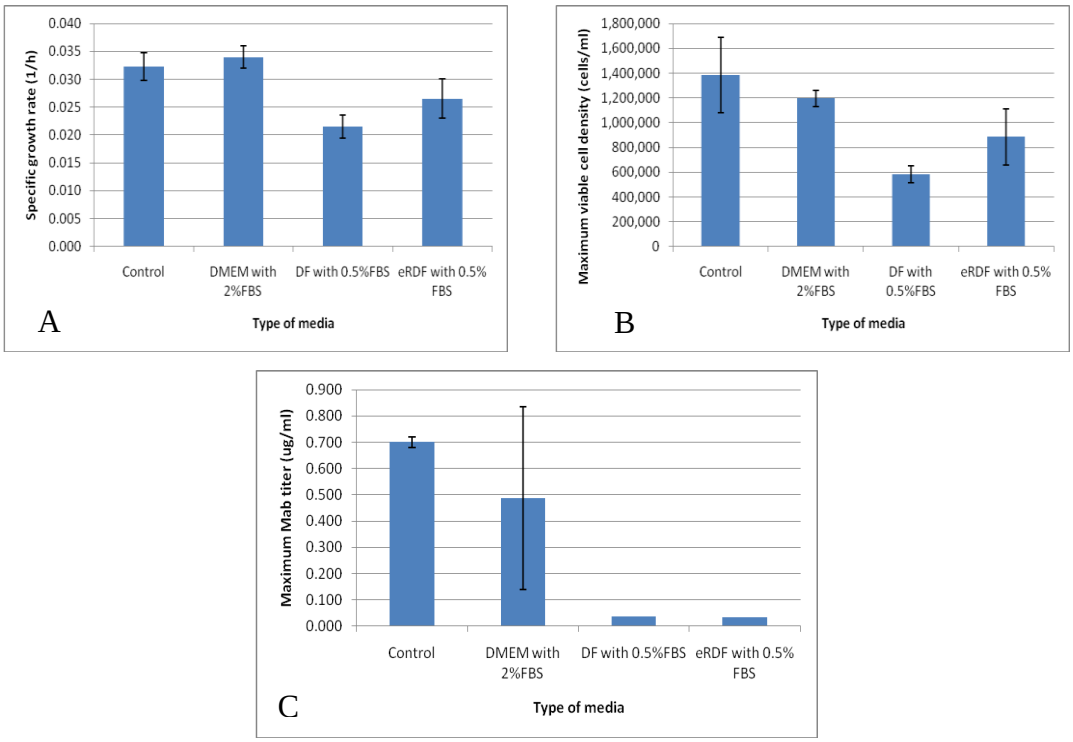


Figure 4.1.2: Comparison of specific growth rates (A), maximum viable cell density (B) and maximum MAb titer (C) for cells cultured in different media

The basic supplements provided by the basal medium seemed incapable of furnishing hybridoma 192 with the required nutrients for growth and production. Nonetheless, hybridoma 192 was able to grow in low serum containing media. This was dependent on the type and number of nutrients available in the medium. For media with

lots of amino acids, vitamins and minerals such as DF and eRDF, cells could survive even with FBS supplementation level of only 0.5%. However, DMEM that contained fewer nutrients was unable to support the growth of hybridoma 192 at low serum content. Even though hybridoma 192 could grow slowly in DF and eRDF supplemented with only 0.5% FBS, the maximum viable cell density that could be achieved was low and the MAb titer obtained at the end of the culture was greatly reduced relative to control. All nutrients appeared to be channeled by the cells for growth and maintenance rather than MAb production, as the viability in these two media was very high at up to 98% (data not shown), but the MAb titer was barely detectable. Since MAb was the product of interest in this study, DMEM supplemented with 2% FBS was chosen as the culture medium for the subsequent work.

4.1.4.2 Glutamine degradation rate in the culture media

As discussed in Section 2.4.6, glutamine tends to degrade chemically. Since the degradation rate is dependent on the composition of medium, the pH and temperature of culture (Ozturk and Palsson, 1990b; Schneider *et al.*, 1996), it has to be determined whenever there is a change in the composition of medium. Ozturk and Palsson (1990b) had shown that an error as large as 200-300% would be made in calculating specific glutamine consumption and specific ammonia production rates if the chemical degradation of glutamine was ignored.

It can be seen from Figure 4.1.3 that glutamine decomposition rates were higher at higher temperature. Glutamine was totally degraded in 26 days (624 h) for DMEM supplemented with 5% FBS and also DMEM supplemented with 2% FBS at 37°C, but the same medium when stored at 4°C did not experience significant loss of glutamine if the storage period was less than a month. Nevertheless, decomposition still occurred at a slower rate. This revealed that glutamine degradation rates were highly dependent on the temperature of storage, as also reported by Ozturk and Palsson (1990b).

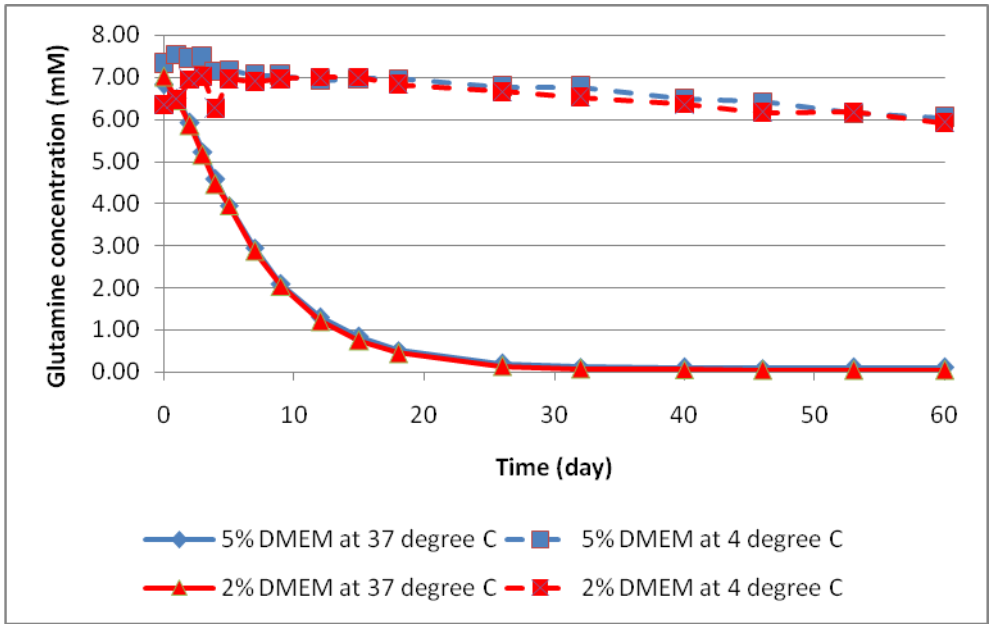


Figure 4.1.3: Glutamine degradation in DMEM supplemented with 5% FBS and 2% FBS at 4°C and 37°C

Glutamine degradation obeyed first-order kinetics and therefore the plots of ln $[Gln]/[Gln]_0$ versus time were linear (Figure 4.1.4). The degradation rate constants (k) calculated from slopes of the plots (Figure 4.1.4) are shown in Table 4.1.1.

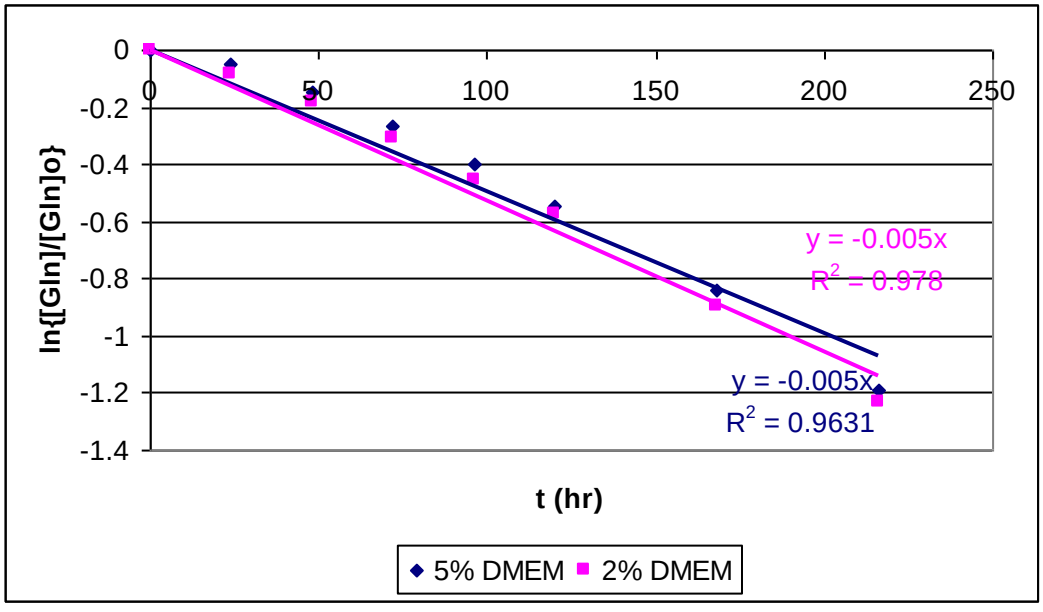


Figure 4.1.4: A plot of ln{$[Gln]/[Gln]_0$} versus t in DMEM + 5% FBS and DMEM + 2% FBS at 37°C ([Gln] is glutamine concentration at time t; $[Gln]_0$ is glutamine concentration at time zero)

Table 4.1.1: Comparison of glutamine decomposition rate constant at 37°C in different media

Type of media	Glutamine decomposition rate constant, *k* (1/h)
5% DMEM	**0.005**
2% DMEM	**0.005**

At 37°C, *k* value for DMEM supplemented with 5% FBS was similar to the *k* value of DMEM supplemented with 2% FBS. This revealed that differences in serum content in the media did not affect the chemical decomposition rate of glutamine. This observation is consistent with the results of Ozturk and Palsson (1990b). However, the *k* value obtained by Ozturk and Palsson (1990b) in DMEM without serum was 0.0025/h at pH 7.7 and 37°C, but the *k* value obtained in this study was 0.005/h at pH 7.7 and 37°C at both serum content. Another researcher, Lin and Agrawal (1988) in their earlier study using DMEM supplemented with 5% serum, obtained a *k* value of 0.0022/h at pH 7.7 and 35.5°C, close to the value of Ozturk and Palsson (1990b) although the temperature used by Lin and Agrawal (1988) was different. The result obtained here is in agreement with Glacken *et al.* (1986) [*k* = 0.0048/h]. Glacken *et al.* (1986) measured *k* at 37°C in DMEM with 5% serum. The pH employed was not specified.

Half-life of glutamine in DMEM at both serum contents was found to be approximately 6 days (Figure 4.1.3). In contrast, the half-life obtained by Lin and Agrawal (1988) in DMEM supplemented with 5% serum was about 13 days. The incubation temperature used in the present work (37°C) was a little higher than 35.5°C used by Lin and Agrawal (1988).

The somewhat different result in this study compared to the one obtained by Ozturk and Palsson (1990b) was possibly due to the likely difference in the pH values used during incubation. Ozturk and Palsson (1990b) used a tissue culture flask that was placed in the CO_2 incubator. Thus, the pH during the experiment remained constant in the absence of cells. In contrast, a tightly closed tube was used in this study, where the pH may have changed during the experiment, e.g. sampling, even though the tube was placed in the CO_2 incubator. Therefore, pH likely became more alkaline towards the end of the experiment and the *k* value increased (Lin and Agrawal, 1988). The pH was

inferred from the purple-red color of the medium, which contained phenol red as a pH indicator.

In real culture conditions in a CO_2 incubator, the pH is not really controlled. With the presence of cells, lactate is produced as a waste product as the cells grow. Thus, pH decreases with time. Since *k* value is dependent on pH, it also changes during culture. Therefore, the glutamine consumption and ammonia production rates that are calculated by using a constant *k* value may not be correct during culture. Nevertheless, the *k* value obtained in this study could be used for correction of glutamine consumption and ammonia production rates in a pH controlled culture system such as in a bioreactor.

4.1.4.3 Growth, production and metabolites profile in T-flask, spinner flask and bioreactor

Batch profiles of cells grown in DMEM supplemented with 5% FBS (control) and 2% FBS (2% DMEM) were obtained as described in Section 4.1.3.4. Typical growth profiles of the cells in T-flask (static culture) were similar for the control medium and 2% DMEM (Figure 4.1.5). The maximum viable cell density attained in 2% DMEM was comparable to control (1.25×10^6 cells/mL versus 1.30×10^6 cells/mL). Even though the culture lifespan seemed to be slightly longer in the control culture, this was mainly due to the longer lag phase experienced by the cell in the control culture.

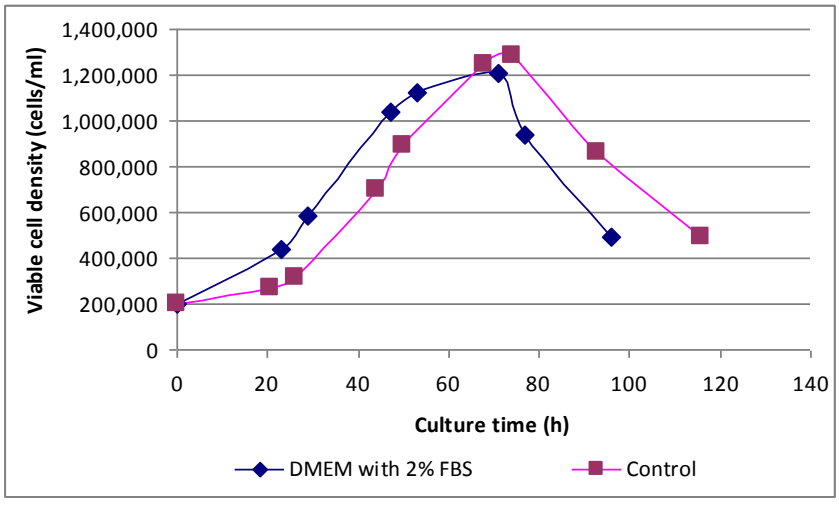


Figure 4.1.5: Typical growth profiles of cells in T-flask culture

The typical growth profiles in spinner flask (stirred culture) are shown in Figure 4.1.6. The profiles for cell growth in both the control medium and 2% DMEM were similar. The only difference was the maximum viable cell density attained. The maximum viable cell density achieved by the control was higher, about 2.10×10^6 cells/mL, as compared to 1.55×10^6 cells/mL that was attained by cells grown in 2% DMEM.

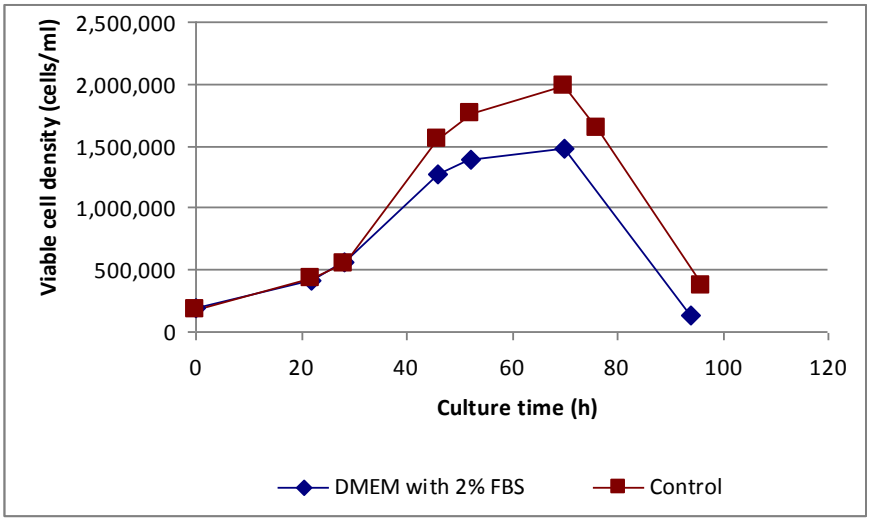


Figure 4.1.6: Typical growth profiles of cells in spinner flask culture

Figure 4.1.7 compares the specific growth rates between control and 2% DMEM in static and stirred cultures. The specific growth rates achieved in both media were comparable in static culture (0.032 ± 0.002/h in control versus 0.036 ± 0.005/h in 2% DMEM). In stirred culture, the specific growth rate in 2% DMEM was slightly lower, but still as good as the values in the control culture (0.041 ± 0.008/h versus 0.047 ± 0.003/h). The higher serum content appeared to have a greater effect on the maximum cell density rather than on the specific growth rate. Serum is known to protect the cells from adverse effects of turbulence in stirred culture (Kunas and Papoutsakis, 1990b; Ozturk and Palsson, 1991b; Michaels *et al.*, 1991; Legazpi *et al.*, 2009). Therefore, higher maximum viable cell density achieved in the control may be possibly due to the higher protective effect at higher serum content. Figure 4.1.7 also reveals that the stirred cultures had significantly higher specific growth rate in both media when compared to the corresponding static cultures. This was apparently due to the improved oxygen supply as a consequence of stirring. Oxygen appears to be one of the limiting nutrients in static culture.

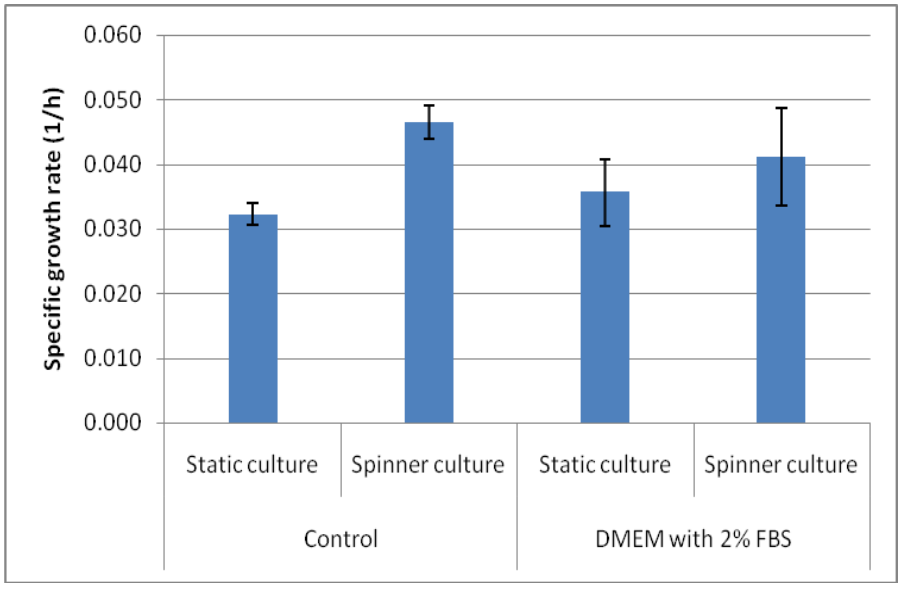


Figure 4.1.7: Specific growth rates of cells in static and stirred cultures

The average maximum MAb titer achieved in the stirred culture was 0.259 µg/mL and 0.184 µg/mL for cells in control culture and 2% DMEM, respectively. These values were much lower than the maximum titer that could be achieved in static culture, which were 0.700 µg/mL (control) and 0.477 µg/mL (2% DMEM). The specific MAb production rates in stirred cultures were also rather low (0.0013 ± 0.0006 pg/cell.h in control and 0.0023 ± 0.0008 pg/cell.h in 2% DMEM) compared to those in static cultures (0.0058 ± 0.0002 pg/cell.h in control and 0.0038 ± 0.0015 pg/cell.h in 2% DMEM; Figure 4.1.8). However, the specific MAb production rate was slightly higher in culture supplemented with only 2% FBS in stirred culture. Apparently, when the growth conditions improved and the growth rate increased, the MAb production rate decreased because of a negatively growth-associated production of MAb. This inverse relationship between growth and specific productivity was consistent with the findings of other researchers (Burky *et al.*, 2007; Suzuki and Ollis, 1990).

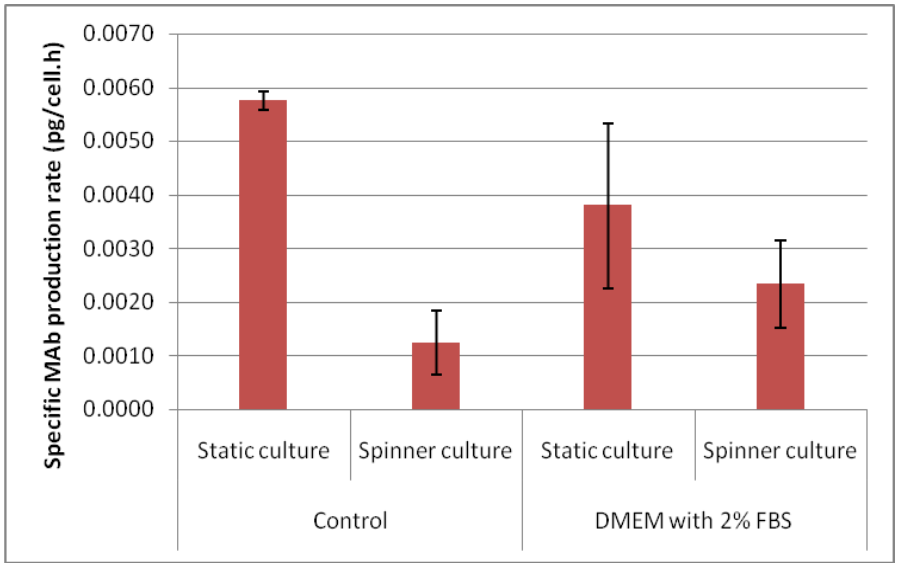


Figure 4.1.8: Specific MAb production rates of cells in static and stirred cultures

Figure 4.1.9 compares the specific metabolites consumption and production rates in static and stirred cultures. Specific lactate production rate was lower in both stirred cultures as compared to static cultures. This was possibly due to improved oxygen supply in stirred cultures that enabled oxidative metabolism instead of anaerobic respiration. Under aerobic respiration, pyruvate produced from glycolysis enters Krebs cycle resulting in reduced lactate production. The flux of glucose through glycolysis is also reduced, resulting in a lower specific glucose consumption rate (McKee and McKee, 2003). Specific glucose consumption rate was indeed reduced by stirring (Figure 4.1.9), but only for the control culture (i.e. DMEM + 5% FBS). For the culture with the lower FBS level, stirring barely had any effect on the specific glucose consumption rate. This concurred with the literature. For example, according to Luo and Yang (2004), when serum content in a medium was reduced, the yield of lactate from glucose also reduced as some glucose was consumed to synthesize the other nutrients that would be otherwise sourced from serum. A reduced yield of lactate on glucose is obvious in Table 4.1.2 with the change in agitation regime from static to stirred in both media.

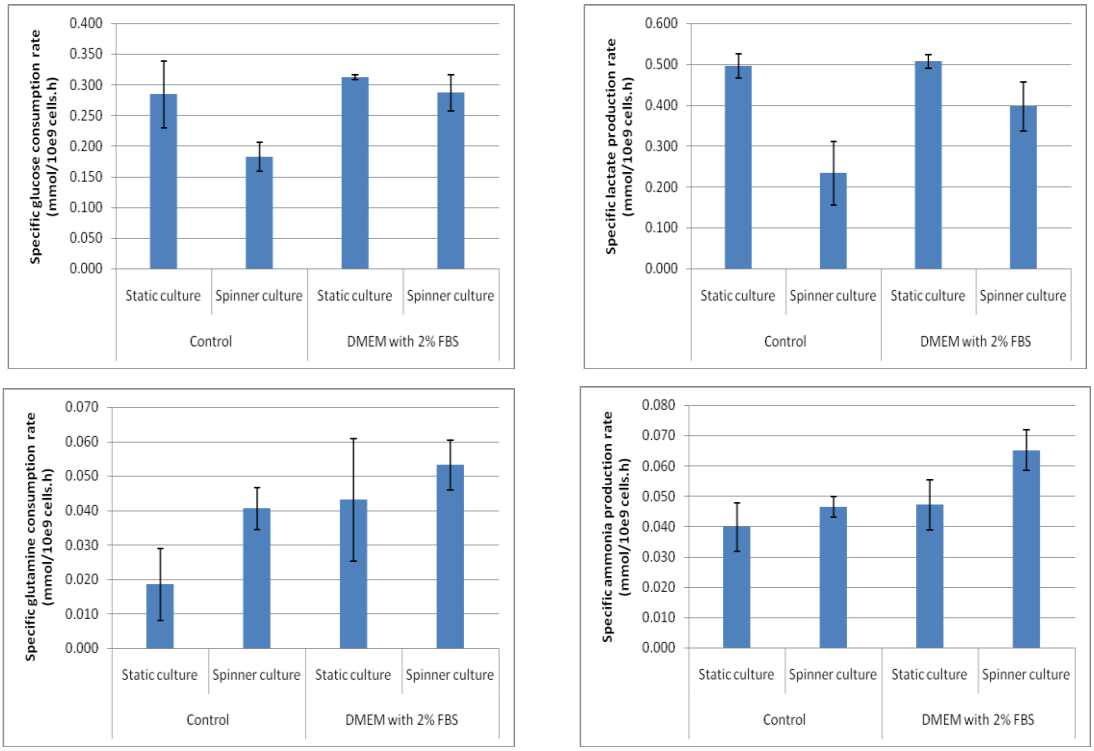


Figure 4.1.9: Specific metabolites production and consumption rates of cells in static and stirred cultures

Table 4.1.2: Comparison of yields in static and stirred cultures

Type of culture	**Static**		**Stirred**	
Serum content	**2%**	**5%**	**2%**	**5%**
Yield of Lac/Glu	1.63	1.75	1.39	1.28
Yield of Amm/Gln	1.09	2.14	1.22	1.15

Figure 4.1.9 showed significant increment of specific glutamine consumption rates and specific ammonia production rates for cells in both media as a consequence of stirring. Under aerobic conditions (i.e. dissolved oxygen ≥ 1.2% of air saturation) increasing glutamine consumption rate with increasing oxygen concentration has been reported (Ozturk and Palsson, 1990a) with an accompanying increase in ammonia production rate. In other cases, for example during rapid growth, increased consumption of glutamine may actually translate into a reduced ammonia yield on glutamine because of incorporation of nitrogen in proteins (Hayter *et al.*, 1992). In this study, the specific glutamine consumption rate increased with stirring (Figure 4.1.9) in agreement with Ozturk and Palsson (1990a). In the low serum medium, the yield of ammonia on

glutamine increased as a consequence of stirring (Table 4.1.2). In contrast, stirring reduced the yield of ammonia on glutamine in the control medium (Table 4.1.2). This difference in behavior could not be ascribed to differences in growth rate as the specific growth rate in low serum media was comparable to that in the control culture (i.e. DMEM + 5% FBS) (Figure 4.1.7).

Figure 4.1.10 illustrates the typical bioreactor growth profile of cells in DMEM supplemented with 2% FBS. The maximum viable cell density was greatly reduced from 1.55×10^6 cells/mL in the spinner flask to about 0.90×10^6 cells/mL in the bioreactor. Specific growth rate of hybridoma 192 was also significantly reduced from 0.041 ± 0.008/h in spinner culture to 0.032 ± 0.005/h in bioreactor (Figure 4.1.11), which was comparable to the value of 0.036 ± 0.005/h in the static culture.

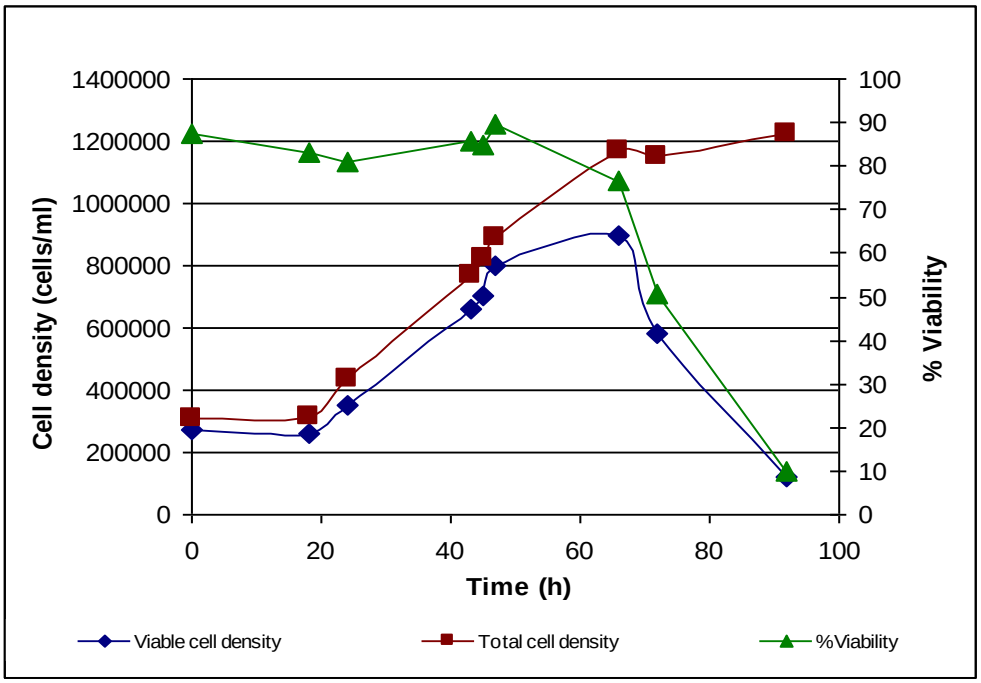


Figure 4.1.10: Typical growth profiles of cells in bioreactor with DMEM supplemented with 2% FBS

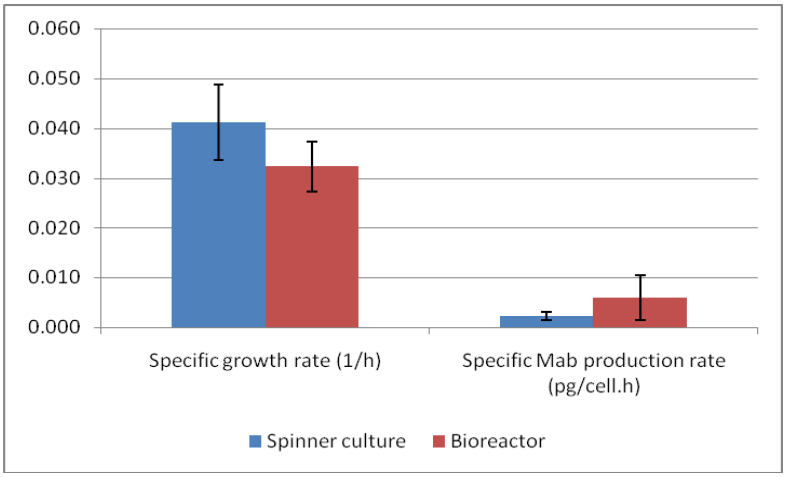


Figure 4.1.11: Comparison of specific growth rate and specific MAb production rate for DMEM with 2% FBS in spinner culture and bioreactor

Figure 4.1.11 also shows the specific MAb production rate in different modes of culture. Specific MAb production rate of hybridoma 192 in the bioreactor (0.0059 ± 0.0045 pg/cell.h) was higher than in the spinner culture (0.0023 ± 0.0008 pg/cell.h). This was in accordance with the negatively growth-associated MAb production characteristics as mentioned earlier. When the specific growth rate of the cells in the bioreactor was reduced because of the stress experienced through stirring and sparging, the specific MAb production rate increased. This was viewed as an indicative trend as the standard deviation of the specific MAb production rate calculated for the bioreactor was rather large.

All the metabolites production/consumption rates were elevated when the cells were cultured in the bioreactor as compared to the spinner culture (Figure 4.1.12). As shown in Table 4.1.3, the yield of lactate from glucose in the bioreactor was comparable to that in the spinner culture, while the yield of ammonia from glutamine was significantly reduced.

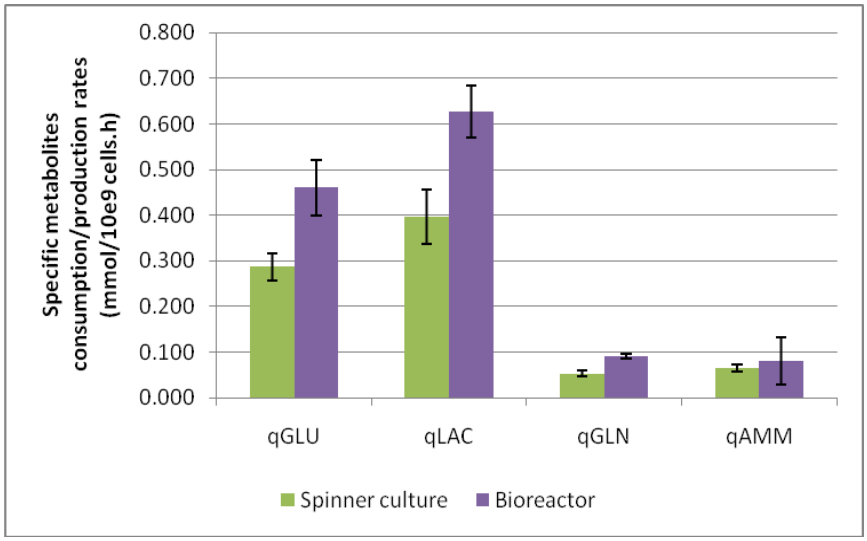


Figure 4.1.12: Comparison of metabolites consumption and productions rates for 2% FBS + DMEM in spinner culture and bioreactor

Table 4.1.3: Comparison of yields in T-flask, spinner culture and bioreactor

Type of culture	T-flask	Spinner culture	Bioreactor
Yield of Lac/Glu	1.63	1.39	1.36
Yield of Amm/Gln	1.09	1.22	0.84

Even though hybridoma 192 was cultured in a better, stirred and fully-controlled environment in the bioreactor as compared to the stirred and uncontrolled environment in the spinner culture, the growth rate was decreased. This may have the following possible explanations. Firstly, hybridoma 192 experienced larger shear effects in the bioreactor. Although the stirring speed employed in spinner culture and bioreactor were both at 75 rpm, the impeller tip speed for spinner culture was 0.196 ms^{-1} compared to 0.212 ms^{-1} in the bioreactor. Therefore, the cells in bioreactor experienced a greater shear force than those in the spinner culture. However, the impeller tip speed calculated based on the diameter of the impeller might not be a proper comparison parameter as the impeller in the spinner culture was a two pendulum system (spaced 30º apart while static; Figure B2), but the stirrer in the bioreactor was a marine type impeller (Figure B6). Secondly, the more violent conditions in the bioreactor as a consequence of sparging. Oxygen was transferred to the medium through diffusion from the headspace in the spinner culture, but oxygen was supplied to the bioreactor through sparging. Sparging has been previously shown to be one of the major causes of cell damage in

mammalian cell cultivation in bioreactors (Kilburn and Webb, 1968; Glacken *et al.*, 1983; Chisti, 2000). In addition, a sparging rate of 0.5 L/min in this case was equivalent to an aeration rate of 0.4 vvm, which was slightly higher than the normal aeration rates employed in mammalian cell culture using bioreactor. Typical aeration rate employed range from 0.007 vvm to 0.15 vvm (Oh *et al.*, 1992; Michaels *et al.*, 1996; Lavery and Nienow, 1987). Higher than normal aeration rate was used in this case because of two reasons: 1) due to the limitations of the equipment in which the controlable range of air flow rates was high; and 2) to test the susceptibility of this cell line to a relatively high level of hydrodynamic shear stress.

In the bioreactor, more energy was apparently channelled for maintenance of cells rather than for growth. This was supported by the apparently increased specific metabolites consumption/production rates (Figure 4.1.12). The pH was fully-controlled at 7.4 in the bioreactor but not controlled at all in the spinner culture. Therefore, pH in the spinner culture varied with time. These changes in pH might, at some point, have exposed the cells to their optimum growth pH and therefore may have increased the observed specific growth rate in the spinner. As discussed in Section 2.4.2, each cell line has its optimum pH for growth and this depends on the medium and the method of cultivation. Hence, the controlled pH 7.4 might not be the optimum pH for growth of hybridoma 192 in the bioreactor even though this was the initial pH of the medium in the static culture. Under stress conditions in bioreactors, cells are known to respond by increasing their rate of metabolism (Oh *et al.*, 1992). This is in accordance with the results obtained in this study.

In an extended duration culture, the slower growing subpopulation of cells may be lost through natural selection that favors the fast growing cells. Since the MAb production is negatively growth-associated, a slow growing population is also the higher producer. Cells that were repeatedly grown as stock in 2% DMEM adapted to this medium. Overtime therefore the cell stock apparently became less productive for MAb. These changes were not suspected until the batch profiles of the hybridoma 192 were measured again. Hence, the low producers may have been unconsciously utilized in the medium optimization study in the following section. The different growth curves (4 runs including duplicates) are shown in Figure 4.1.13.The specific growth rate, the MAb production and the metabolites production/consumption rates are compared in

Table 4.1.4. The maximum viable cell density achieved was a little higher in the current cell stock of hybridoma 192 in 2% DMEM as compared to the old cell stock (1.40×10^6 cells/mL versus 1.25×10^6 cells/mL). In addition to having a higher specific growth rate, the new stock had a somewhat higher specific MAb production rate. Similarly, specific glucose consumption, specific glutamine consumption and specific ammonia production rates were elevated. In contrast, specific lactate production rate showed no significant changes. Apparently, the differences in the growth, production and metabolites specific rates of the cell stocks were due to the number of passages since the cells were first adapted in 2% DMEM rather than any natural selection. The current cell stock was from a higher number of passages, thus appeared to be well adapted to 2% DMEM. On the other hand, the old cell stock was from a lower number of passages, which meant that the cells may not have fully adapted when the experiments were carried out. The data for comparison with the following sections will refer to the data from old cell stock (Table 4.1.4) since the cell stocks used for the following study were from the early number of passages.

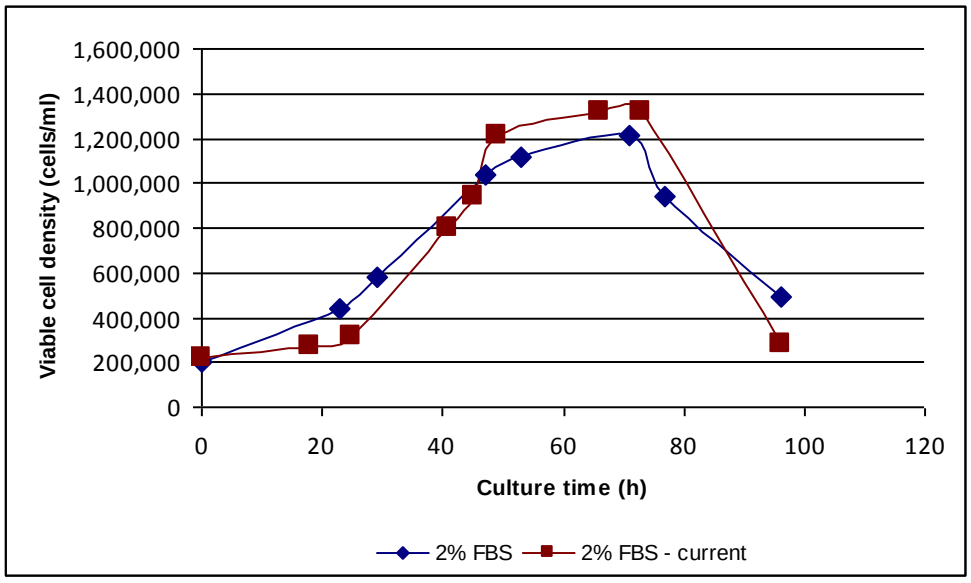


Figure 4.1.13: Comparison of growth profiles for old and new cell stock in 2% DMEM in static culture

Table 4.1.4: Comparison of specific growth, specific MAb production and specific metabolites rates for old and new cell stock in 2% DMEM at static culture

Cell stock	Old	New
Specific growth rate, μ (1/h)	0.036 ± 0.005	0.045 ± 0.003
Specific MAb production rate, r (pg/cell.h)	0.0038 ± 0.0015	0.0043 ± 0.0021
Specific glucose consumption rate, qGLU (mmol/10^9 cells.h)	0.312 ± 0.004	0.324 ± 0.003
Specific lactate production rate, qLAC (mmol/10^9 cells.h)	0.507 ± 0.017	0.455 ± 0.078
Specific glutamine consumption rate, qGLN (mmol/10^9 cells.h)	0.043 ± 0.018	0.058 ± 0.013
Specific ammonia production rate, qAMM (mmol/10^9 cells.h)	0.047 ± 0.008	0.058 ± 0.000

4.1.4.4 Fed-batch study in spinner flask

A simple feeding strategy was used to feed the culture in order to extend the culture lifespan. A fresh medium was fed to a final volume of 230 mL during mid log phase, that is when the viable cell density exceeded 1×10^6 cells/mL or at 42 – 50 h of culture. The initial volume was 90 mL and the stirring speed was 40 rpm. From Figure 4.1.14, it can be seen that the cells grown in DMEM supplemented with 5% FBS (control culture) achieved a higher total maximum viable cell number of 470×10^6 cells as compared to cells grown in DMEM supplemented with 2% FBS (~ 325×10^6 cells on average). Both values were higher than those attained in the batch culture, where the total maximum viable cell number achieved were 173×10^6 cells and 116×10^6 cells for the media with 5% FBS and 2% FBS, respectively. Feeding fresh medium during mid exponential phase appeared to extend the culture lifespan and increase the total maximum viable cell number that could be attained (compared to batch culture as in Figure 4.1.6). A similar observation was reported by other researchers (Reuveny *et al.*, 1986a; Ljunggren and Häggström, 1990; Bushell *et al.*, 1994; Altamirano *et al.*, 2004).

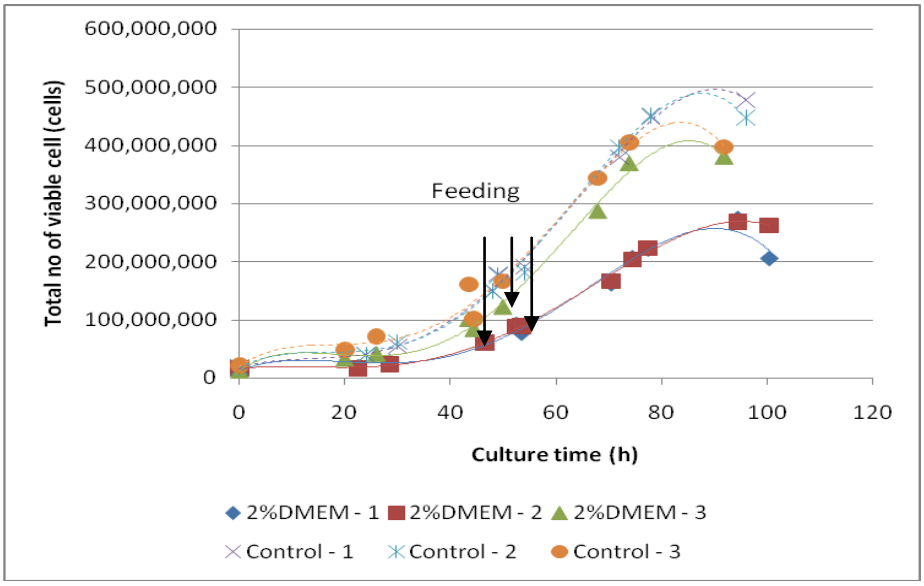


Figure 4.1.14: Growth profiles of cells in spinner flasks for simple fed-batch

In feeding the medium, the volume increased from 90 mL to 230 mL. This affected oxygen transfer to the cells because the surface-to-volume ratio in the spinner changed from 0.526 cm^{-1} to 0.204 cm^{-1}. Thus, although the total final cell count increased because of improved availability of nutrients as noted above, the specific growth rate decreased after the volume changed (Table 4.1.5). A similar trend was observed for the control culture. Thus, oxygen was a critical limiting factor in determining the specific growth rate of this cell line. Improved cell growth in stirred culture was, therefore, due to improved oxygen transfer as compared to static culture.

Table 4.1.5: Comparison of specific growth rates, specific MAb production rates, metabolites consumption and production rates, and yields of cells in 2% and 5% serum media in fed-batch culture

Serum content	**DMEM + 2% FBS**		**DMEM + 5% FBS**	
Stage of culture	*Before feeding (batch)*	*After feeding*	*Before feeding (batch)*	*After feeding*
μ (/h)	0.053 ± 0.002	0.043 ± 0.004	0.055 ± 0.003	0.038 ± 0.010
r (pg/cell.h)	0.00003 ± 0.00002		0.00002 ± 0.00001	

Table 4.1.6 compares the specific growth rates for spinner batch culture at 40 rpm (before feeding in fed-batch culture) and 75 rpm (batch culture from Section 4.1.4.3). Surprisingly, both media afforded a higher specific growth rate at the lower stirring speed. This was possibly due to shear effects at the high stirring speed.

Table 4.1.6: Comparison of specific growth rates of cells in 2% and 5% serum media (DMEM) in spinner flask at different speed for batch culture

Speed	**40 rpm**		**75 rpm**	
Serum content	**2%**	**5%**	**2%**	**5%**
μ (/h)	0.053 ± 0.002	0.055 ± 0.003	0.041 ± 0.008	0.047 ± 0.003

The MAb production rate of the culture in the fed-batch study was rather low (Table 4.1.5). Cells were almost non productive when the medium was fed during the mid log phase as most of the energy and nutrients were diverted for cell growth. Consequently, cell density attained increased but MAb production decreased. This was mainly due to the negatively-growth associated MAb production characteristic of the cell line as observed in the previous section.

4.1.5 Conclusion

Hybridoma 192 was able to adapt to the medium with a serum content as low as 0.5%. However, this was at the expense of specific growth rate and specific MAb production rate. DMEM supplemented with 2% FBS, 1% antibiotic-antimycotic and 4 mM L-glutamine was the best medium that afforded growth and production rates that were comparable to the control culture (i.e. exactly the same medium but with 5% FBS). Culturing cells in the spinner flask improved the specific growth rate relative to static cultures apparently because of improved oxygen supply. This was further supported by the data obtained in the fed-batch mode. Specific growth rate decreased as oxygen transfer into the medium decreased. The fed-batch mode invariably improved the total maximum viable cells number attained in the stirred culture. At the same time, it also extended the culture lifespan. However, specific MAb production rate reduced tremendously. In addition, stirring speed was found to affect growth rate of cells: the higher was the stirring speed, the lower was the growth rate. When the hybridoma 192

was cultured in the fully-controlled environment of the sparged bioreactor, the shear effects of sparging and the high impeller tip speed reduced the growth rate of the cells. Nonetheless, the specific MAb production rate was improved as a consequence of the negatively-growth associated production characteristic. Thus, a mechanism that suppresses growth but maintains viability after the cell density has reached a sufficient number should be developed in order to attain a high MAb productivity.

4.2 Medium Optimization Using Statistical Experimental Design

4.2.1 Introduction

The results in Section 4.1 demonstrated that hybridoma 192 was able to survive without serum if appropriate supplements were provided. However, as discussed in Section 2.3.4, simply adding hormones or growth factors to the medium to compensate for a lack of serum will only add to the cost of MAb production. Thus, the aim was to develop a low serum medium with minimal supplements. Only low-cost supplement were acceptable. This was done by screening the potential supplements and optimizing the medium.

To survive *in vitro*, mammalian cell requires a complicated nutrient environment for growth. The basal media that are typically used in cell culture provide only the basic requirement for survival of the cells. Serum provides the other nutritional requirements. In reduced serum and serum-free media, other components are required to be added to the medium in order to substitute the function of serum. Besides glucose and glutamine, the most common low-cost supplements used in serum- and protein-free media are ethanolamine, selenite, and essential and nonessential amino acids. Amino acids are the main nitrogen source in the media and have been found to promote cell growth (Franěk *et al.*, 2003; Gong *et al.*, 2006; Qi, 1993). Feeding of amino acids during fed-batch culture has allowed cells to maintain a high cell viability for a longer period (Ducommun *et al.*, 2001). In addition, supplementation with amino acids has enhanced antibody production (Ducommun *et al.*, 2001; Gong *et al.*, 2006; Hiller *et al.*, 1994). Furthermore, when leucine, lysine, isoleucine and valine are supplemented at 3 times their normal concentrations, the fraction of amino acid nitrogen that is excreted as ammonia is reduced (Hiller *et al.*, 1994). Certain essential amino acids (e.g. threonine) have been found to protect CHO (Chinese Hamster Ovary) cells from hyperosmolality stress (deZengotita *et al.*, 2002) and ammonium stress (Chen and Harcum, 2005). Cells encounter various types of toxic agents in culture. Therefore, antioxidants are required in the medium in order to protect cell from these reactive agents. Sodium pyruvate and 2-mercaptoethanol are two examples of antioxidants that are typically added to the culture. According to Qi (1993), sodium pyruvate that is present in most of the basal media, reacts rapidly with the hydrogen peroxide generated by the photooxidation of

media components and protect the cells from cytolytic effects. Lipids are required for cell growth, either as an alternative energy source, components of cell membranes, or as precursors for prostaglandin synthesis (Qi, 1993). Therefore, most of the serum-free media are fortified with various types of lipids. In 1982, Murakami and co-workers (1982) identified phosphoethanolamine and ethanolamine as two components that promoted growth. Phosphoethanolamine is converted by cells to ethanolamine before consumption and the concentration required is ten-fold of the ethanolamine concentration. Thus, ethanolamine is preferentially used to substitute lipids by most researchers (Eto *et al.,* 1991; Nagira *et al.,* 1995). However, Qi (1993) has shown that hybridoma cells can be adapted gradually to media without ethanolamine.

Selenium is an essential component in serum-free media. Selenium deficiency has been shown to induce cell death (Saito *et al.,* 2003). It is believed that selenium is a cofactor of glutathione peroxidase which protects the organism from oxidative damage (Baker *et al.,* 1998; Qi, 1993; Saito *et al.,* 2003). Zhang *et al.* (2006) found that selenium is an iron carrier for CHO cell line. Thus, it may work in the same manner in other cell lines. Typical form of selenium added into serum-free media is sodium selenite. A few researchers have shown that supplementing culture with sodium selenite enhances cell growth rates (Eto *et al.,* 1991; Murakami *et al.,* 1982; Qi 1993; Zhang *et al.,* 2006). Transferrin is also added to most of the serum-free media. Yet, it typically comes from animal sources. Therefore, to develop protein-free media, a substitute that has similar function as transferrin in the media is required. Iron salts are able to replace the role of transferrin in serum- or protein-free media (Eto *et al.,* 1991; Kovář and Franěk, 1987; Nagira *et al.,* 1995; Qi, 1993; Zhang *et al.,* 2006). A range of iron salts have been tested, but the one commonly used and the most promising appears to be ferric citrate (Eto *et al.,* 1991; Kovář and Franěk, 1987; Nagira *et al.,* 1995). It has been shown to both stimulate growth and MAb production. The concentration of ferric citrate typically used ranges from 8 µM to 3.6 mM (Eto *et al.,* 1991; Nagira *et al.,* 1995; Qi 1993; Zhang *et al.,* 2006). Insulin is also another component that has long been used as a growth and maintenance factor in serum-free media. However, it is an animal protein. Wong *et al.* (2004) have demonstrated that zinc sulfate can be used to replace insulin in mammalian cell culture media. No adaptation was required when hybridoma cells were supplemented with 1.5 mg/L of zinc sulfate to replace insulin in serum-free media (Wong *et al.,* 2004). Zhang *et al.* (2006) used 0.43 mg/L of zinc sulfate, together with

some other components in a protein-free medium, to successfully enhance growth and production of CHO cells. Even though Eto *et al.* (1991) quoted that hybridoma cells do not require insulin and no replacement is required when developing protein-free media, zinc sulfate will be considered in this study. This is because zinc is also a trace element, which is essential for all types of living matter. Thus, when serum is not present in the protein-free media, it may be necessary to add some extra trace elements to the culture. In view of the above, development of a simple protein-free medium such as the one used by Wong *et al.* (2004) is possible without sacrificing productivity in at least some cell lines. Although these supplements might be able to improve the maximum cell density and MAb productivity of the hybridoma cell line in this study, they greatly add to the cost of the medium. A diagnostic antibody needs to be inexpensive especially when other competing methods exist for diagnosis of a disorder such as CAH. This study will therefore focus on using mainly variants of the existing formulations of basal media, possibly supplemented with inexpensive nutrients (e.g. iron salts to replace transferrin, zinc to replace insulin; glucose; glutamine) in attempts to optimize the growth and production.

The conventional method of media development involves screening of a large number of components and optimizing them one at a time. This method is laborious and time consuming. Most importantly, by studying one component at a time, a researcher may miss the possible interactions between the components that influence the output responses. Thus, statistical experimental design, which investigates the mathematical relationships between the input variables and the output responses, is a preferred approach to medium development. Design of experiment methodology is an effective way to optimize media with a reduced number of experiments (Mandenius and Brundin, 2008). Design of experiment methodology has been extensively used for development and optimization of media for microbial production process, but less so for processes involve animal cells.

4.2.2 Objectives of study

The present work aimed at developing and optimizing a low serum or serum-free medium for the cultivation of hybridoma 192 cells in producing an antibody for possible use in diagnosis of CAH. The goal was to substitute serum with a minimum number of low-cost protein-free components, such that the resulting medium supports growth and MAb productions level that are comparable to, or better than, the results obtained in DMEM supplemented with 2% FBS.

4.2.3 Materials and methods

4.2.3.1 Supplements

All supplements (ferric citrate, ethanolamine, 2-mercaptoethanol, zinc sulfate, essential amino acids and sodium selenite) were purchased from Sigma Chemicals, St. Louis, MO, USA. Stock solutions of the supplements (except essential amino acids; M5550, Sigma) were prepared as indicated in Table 4.2.1. The concentrations of each supplement employed in the experiments are shown in Table 4.2.1. The media (10 mL each) with various supplements were prepared following the recipe indicated in the experimental design (Table C18, Appendix C).

Table 4.2.1: Concentrations of components in the experiments and stock solutions

Component	Concentration range used in the experiment	Concentration in stock solution
Essential amino acids	0 - 2% (v/v)	-
2-Mercaptoethanol	0 – 4 µL/L	2400 µL/L
Ethanolamine	0 – 3 µL/L	1800 µL/L
Ferric citrate	0 – 500 µM	300 mM
Zinc sulfate	0 – 6 µM	3.6 mM
Sodium selenite	0 – 25 nM	30 µM

4.2.3.2 Statistical experimental design

Screening experiment was designed by using Design Expert version 7. A 2-level factorial design was chosen for the screening purpose. 2^{6-1}_{V} design (The subscript V here means a high resolution design that was able to estimate the main and the 2-factor interactions) was selected with 2 replicates. Because cell counts are slow, experiments were carried out in blocks of 4. Each block contained 17 runs, in addition to a control culture. One center point was allocated per block so as to check for curvature and error of experiments. The experimental runs (Table C18, Appendix C; runs 1 – 68) were generated based on the high and low levels of concentrations of each component as shown in Table 4.2.1. Four center points (runs 69 – 72, Table C18) were added in separate blocks to increase the accuracy and reduce the error.

4.2.3.3 Cell culture procedures

All cultures were carried out in 6-well plates (Nunc, Denmark) with a total volume per well of 2.5 mL. Inoculum density in each block of experiments was in the range of 1.8 – 3.5 × 10^5 cells/mL. One control culture (basal medium + 2% v/v FBS) was run together with each block. The plates were incubated at 37°C with 5% CO_2 in a humidified incubator (Shellab, Sheldon Manufactring Inc., USA). A 1.5 mL sample was taken after 65 h of culture for cell counts and the supernatant was kept at -20°C for future analyses. The storage period did not exceed 60 days.

4.2.3.4 Augmentation to central composite design

Two-level fractional factorial design was augmented to central composite design by using Design Expert version 7. Faced centered design was chosen. Extra 18 runs were added to the design, where 6 of them were center points. Each factor was varied between the minimum and maximum (Table 4.2.1), one at a time, in the rests of the runs, in which all other factors remained at their center point values (runs 73 – 90, Table C18). Culture procedure was exactly the same as described for the screening experiment.

4.2.3.5 Adaptation to new medium

A slow adaptation process was used. Thus, the cells were first adapted into the new medium, by mixing the new medium (NM) and DMEM supplemented with 2% FBS in 4:1 (v/v) ratio, at an inoculum density of 2×10^5 cells/mL. Cell count was done every 24 h and cells were subcultured every 48 h. Once the viability had exceeded 90%, the cells were further adapted to NM containing 0.1% FBS or a totally serum-free NM. When the growth rate had returned to normal and the viability was greater than 90%, the cells were considered adapted in a new medium.

4.2.3.6 Validation process in new medium

Cells from mid-log phase (viability > 90%), which had already adapted to the new medium, were centrifuged and re-suspended in fresh medium at an inoculum density of 2×10^5 cells/mL. Culture was run in duplicate in T25 flasks. A duplicate culture of cells in control medium (basal media + 2% v/v FBS) was run in parallel. A 0.5 mL sample was taken after 24 h and twice daily subsequently.

4.2.4 Results and discussion

4.2.4.1 Screening of the significant factors

Six components were screened for their ability to support growth of hybridoma 192 in DMEM with very low or zero serum supplementation. This aimed to optimize the medium for culturing hybridoma 192 with minimal supplements. The experiment was design as described in Section 4.2.3. Statistical analysis of the results for the screening experiment are shown in Table 4.2.2 and Table 4.2.3 for the experimental responses (viable cell density, % viability and MAb titer), obtained from the 72-runs of the 2-level factorial experiment (Table C18, Appendix C). Variables with a positive effect are beneficial to the corresponding responses and the other way round for variables with a negative effect. As revealed in Table 4.2.2 and Table 4.2.3, curvature was significant for all three responses. As a result of the inability to determine which

variables contributed to the curvature, the experiment was augmented to a central composite design.

Table 4.2.2: Analysis of variance table for cell density and percentage cell viability for the screening design

Factors	Viable cell density			% Viability		
	Effect	**F-ratio**	**p-value**	**Effect**	**F-ratio**	**p-value**
A-MEM amino acids	0.016	0.20	0.6570	-0.015	0.053	0.8194
B-2-mercaptoethanol	-0.27	56.84	< 0.0001*	-0.67	110.12	<0.0001*
C-Ferric citrate	0.49	183.51	< 0.0001*	0.92	208.85	<0.0001*
D-Ethanolamine	-0.12	10.34	0.0024*	-0.18	8.21	0.0063*
E-Sodium selenite	0.069	3.68	0.0617	0.12	3.66	0.0620
F-Zinc sulfate	0.30	66.51	< 0.0001*	0.68	113.72	<0.0001*
AD	0.078	4.63	0.0370*	0.98	3.79	0.0578
BC	0.21	34.08	<0.0001*	12.14	47.16	<0.0001*
BD	0.073	4.08	0.0495*	1.82	7.06	0.0109*
BE	0.079	4.76	0.0345*	1.15	4.48	0.0398*
CF	0.076	4.44	0.0409*	3.01	11.68	0.0013*
ABE	-0.12	11.45	0.0015*	3.38	13.14	0.0007*
BDE	0.086	5.58	0.0227*	1.36	5.28	0.0263*
DEF	0.38	6.48	0.0145*	-	-	-
Curvature	-	68.94	**<0.0001***	-	65.02	**<0.0001***

* Significant at $p < 0.05$

Table 4.2.3: Analysis of variance table for MAb titer for the screening design

Factors	Effect	F-ratio	p-value
A-MEM amino acids	0.0015	0.28	0.5965
B-2-mercaptoethanol	-0.011	16.03	0.0002*
C-Ferric citrate	0.0011	0.16	0.6912
D-Ethanolamine	0.0079	7.88	0.0069*
E-Sodium selenite	0.0041	2.08	0.1549
F-Zinc sulfate	0.0068	5.74	0.0199*
AD	0.0071	6.34	0.0147*
BD	0.0061	4.73	0.0339*
CF	0.0066	5.43	0.0235*
DE	-0.0057	4.03	0.0495*
Curvature	-	21.91	**<0.0001***

* Significant at $p < 0.05$

4.2.4.2 Augmentation to central composite design (CCD)

The whole design was converted to a response surface model and an extra 18 runs were added in order to identify and optimize the significant factors that contributed to the curvature. Table 4.2.4 reveals that among the four significant factors at a confidence level $p < 0.05$ for both cell density and percentage of cell viability, only ferric citrate and zinc sulfate had a positive effect. Sodium selenite had a positive significant effect at a confidence level $p \leq 0.1$ for both responses. Table 4.2.5 shows the goodness of fit (R^2) and the goodness of prediction (Q^2) of the models obtained. According to Mandenius and Brundin (2008), models with a R^2 of greater than 0.75 and Q^2 of greater than 0.60 are normally designated as good models, whereas model with a R^2 or Q^2 value of below 0.25 are considered useless. Since the main objective of this screening and optimization of medium was to reduce serum dependency of the hybridoma cells and enhance cell growth, the relevant models (Eq. 4.2.1 and Eq. 4.2.2) were considered satisfactory, but the model for MAb titer (not shown) was discarded as it had a relatively low values of R^2 and Q^2 (Table 4.2.5).

Table 4.2.4: Analysis of variance table for cell density and percentage cell viability for the augmented design

Factors	Viable cell density			% Viability		
	Effect	**F-ratio**	**p-value**	**Effect**	**F-ratio**	**p-value**
A-MEM amino acids	0.019	0.20	0.6579	-0.16	0.026	0.8715
B-2-mercaptoethanol	-0.27	39.79	< 0.0001*	-7.75	63.82	<0.0001*
C-Ferric citrate	0.50	141.63	< 0.0001*	11.87	149.76	<0.0001*
D-Ethanolamine	-0.11	6.46	0.0138*	-2.79	8.27	0.0057*
E-Sodium selenite	0.074	3.13	0.0822	1.58	2.66	0.1081
F-Zinc sulfate	0.30	52.14	< 0.0001*	9.95	105.24	<0.0001*
BC	0.21	24.48	<0.0001*	4.41	20.07	<0.0001*
BD	-	-	-	2.35	5.68	0.0205*
CF	-	-	-	3.91	15.78	0.0002*
DE	-	-	-	-2.23	5.15	0.0271*
C^2	-0.70	10.00	0.0025*	-18.55	13.08	0.0006*

* Significant at $p < 0.05$

Table 4.2.5: R^2 and Q^2 of the models

Responses	**Viable cell density**	**% Viability**	**MAb titer**
Goodness of fit, R^2	0.7912	0.8360	0.3561
Goodness of prediction, Q^2	0.6207	0.6981	-0.1930

The experimental results obtained from CCD were fitted to a quadratic model, giving two numerical correlations in terms of coded factors as follows:

$$\ln(\text{cell density}) = 13.93 + 0.019A - 0.27B + 0.50C - 0.11D + 0.074E + 0.30F - 0.041AB - 0.003AC + 0.078AD - 0.001AE + 0.032AF + 0.21BC + 0.073BD + 0.079BE - 0.018BF - 0.012CD - 0.049CE + 0.076CF - 0.067DE - 0.022DF - 0.005EF + 0.12A^2 - 0.005B^2 - 0.70C^2 - 0.15D^2 - 0.10E^2 - 0.41F^2$$

Eq. (4.2.1)

$$Viability = 73.90 - 0.16A - 7.75B + 11.87C - 2.79D + 1.58E + 9.95F - 0.77AB + 0.33AC + 1.69AD + 0.21AE + 0.031AF + 4.41BC + 2.35BD + 1.54BE - 1.23BF - 0.90CD - 0.76CE + 3.91CF - 2.23DE - 1.50DF - 0.22EF + 3.85A^2 + 3.05B^2 - 18.55C^2 - 4.10D^2 - 2.00E^2 - 11.90F^2$$

Eq. (4.2.2)

The analysis of variance for the CCD experiments gave relatively high F values (12.79 for cell density and 16.86 for viability), very low probability values (<0.0001 for cell density and <0.0001 for viability), and fairly large goodness of fit (Table 4.2.5). These were all indicative of the good fit of the models. By combining Eq. (4.2.1) and Eq. (4.2.2), Design Expert 7.0 estimated the optimum point with the following concentrations of supplements: ferric citrate = 311.79 µM, sodium selenite = 17.28 nM and zinc sulfate = 4.47 µM. At this optimum point the maximum estimated cell density was 1.92×10^6 cells/mL and the viability was 96% (Figure 4.2.1 and Figure 4.2.2).

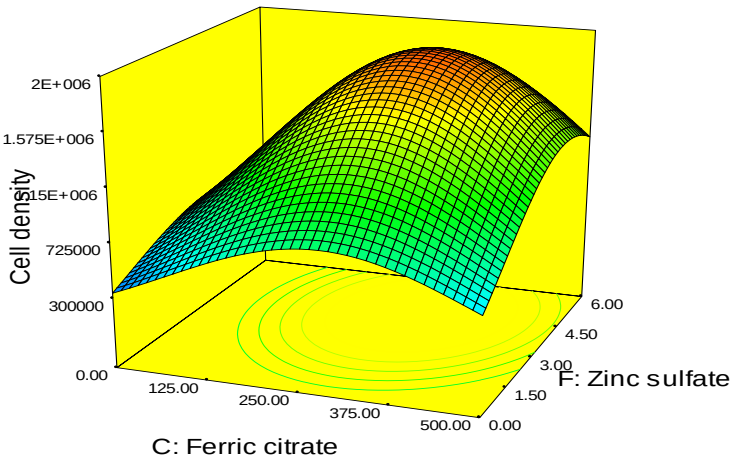


Figure 4.2.1: 3D plot showing optimum point for viable cell density at 17.28 nM sodium selenite

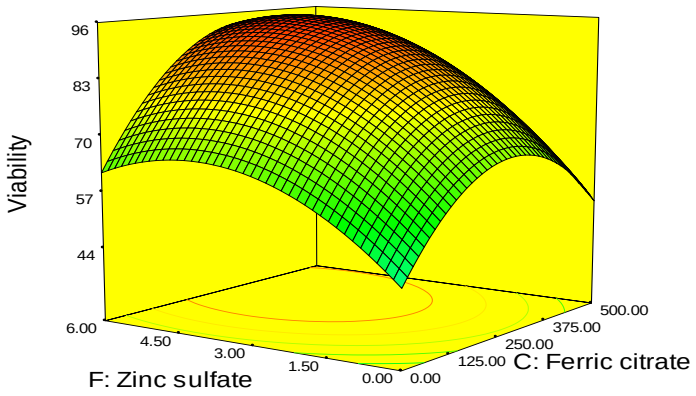


Figure 4.2.2: 3D plot showing optimum point for % viability at 17.28 nM sodium selenite

4.2.4.3 Adaptation to new medium

Before a validation run at the above predicted optimal conditions, the cells needed to be adapted to the above new medium formulation (NM). Figure 4.2.3 shows the growth profile during the adaptation process in the NM with the final serum content of 0.4% and 0.1%. The adaptation process was run twice and similar results were obtained. Figure 4.2.3 clearly shows that hybridoma 192 cells were able to adapt to NM supplemented with 0.4% FBS as repeated good growth and high viability were achieved. Unfortunately, a further reduction in the serum content to 0.1% adversely affected cell growth and after two subculturing all cells experienced apoptotic cell death (Apoptotic death was evidenced by microscopic observations of shrunken cells with chromatin condensation.). Therefore, a further reduction of serum level to less than 0.4% was not feasible. The complete new medium formulation (Low Serum DMEM or LSD) thus contained DMEM supplemented with 0.4% FBS, 4 mM L-glutamine, 1% antibiotic-antimycotic, 0.1% Pluronic F68, 311.79 µM ferric citrate, 17.28 nM sodium selenite and 4.47 µM zinc sulfate.

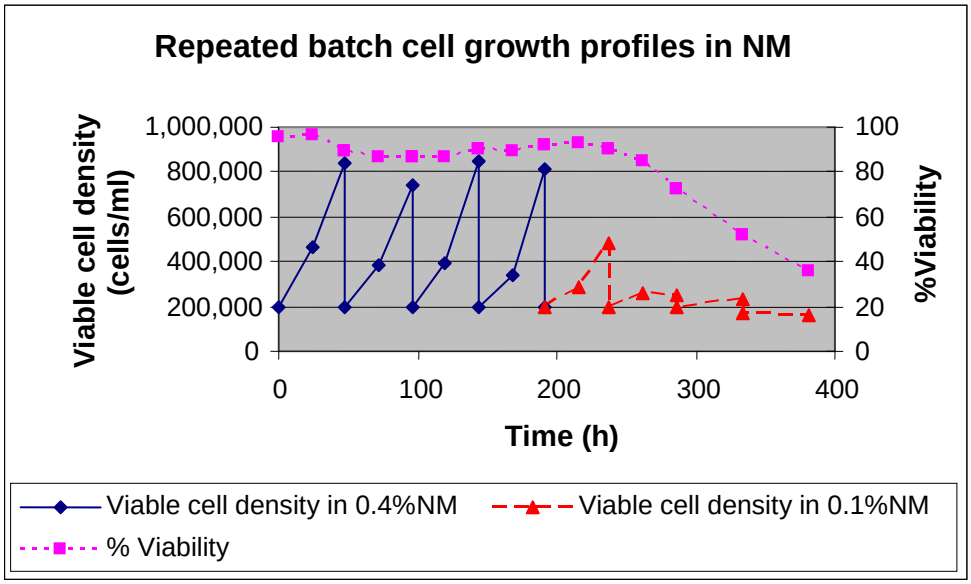


Figure 4.2.3: Repeated batch cell growth profile in NM (2nd trial)

Cells grown in NM supplemented with 0.4% FBS (or LSD) were expanded into spinner culture and then preserved as a master cell stock. Cells preserved in this medium were found to withstand repeated freezing and thawing.

4.2.4.4 Batch profile for cells growing in LSD

Figure 4.2.4 shows the growth and production profiles of cells in LSD in the validation experiment. The cells grown in LSD had a slightly longer culture lifespan than those in the control culture (2% DMEM). This may have been simply due to the longer lag phase experienced by the cells grown in LSD. The maximum viable cell density attained was about 1.55×10^6 cells/mL, comparable to the maximum viable cell density achieved in the control (1.7×10^6 cells/mL), but lower than the predicted value of 1.92×10^6 cells/mL. The MAb titer attained at harvest in the LSD medium was 1.264 µg/mL compared to 0.435 µg/mL in the control culture (Figure 4.2.4). As shown in Table 4.2.6, the specific growth rate for the cells was lower in LSD than in the control culture. The specific MAb production rate, on the other hand, was higher in LSD culture compared to control culture (Table 4.2.6). The stress experienced by the cells due to the low serum content in the LSD may have contributed to the enhancement of the MAb productivity. As a result of a stressful environment, the cells had a reduced growth rate and more energy could be diverted to production of MAb. This was consistent with a negatively growth-associated production characteristic.

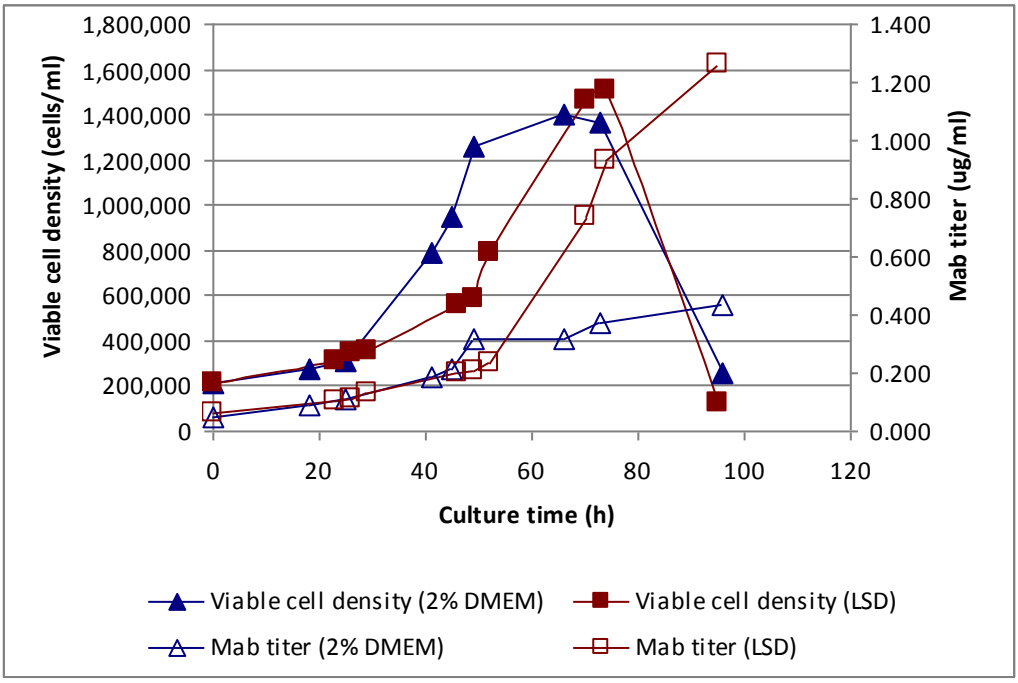


Figure 4.2.4: Growth and production profiles for cells grow in LSD

Table 4.2.6: Comparison of specific growth rates and MAb production rates of cells growing in LSD and control

Type of medium	Specific growth rate (1/h)	Specific MAb production rate (pg/cell.h)
LSD	0.033 ± 0.001	0.0569 ± 0.0453
DMEM + 2% FBS	0.045 ± 0.003	0.0043 ± 0.0021

The results revealed that inexpensive supplements added in relatively low concentrations to a DMEM basal medium could reduce the serum requirement of the cells. As shown in Table 4.2.7, the cost of the redesigned medium was nearly 70% lower than the cost of the original medium (i.e. DMEM + 2% FBS). This will certainly reduce the cost of production of the antibody in any future manufacturing process. In addition, the reduced serum level of 0.4% should facilitate the downstream purification of the antibody.

Table 4.2.7: Cost reduction of the medium

Type of medium	Total cost per L of medium in RM*
Control	132.15
LSD	39.03
Percentage of cost reduction	***70.5%***

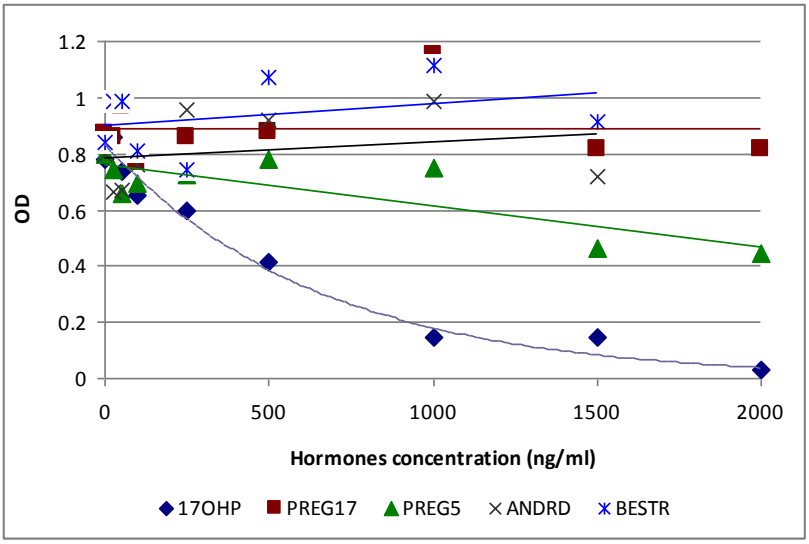
* Calculated based on the local price in 2007

Ferric citrate is believed to stimulate both growth and MAb production. It is found to be able to substitute transferrin in serum- or protein-free media (Eto *et al.*, 1991; Kovář and Franěk, 1987; Nagira *et al.*, 1995; Zhang *et al.*, 2006). Zinc sulfate has been claimed as a satisfactory substitute for insulin in serum-free media (Wong *et al.*, 2004; Zhang *et al.*, 2006). Selenium acts as an antioxidant that protects cells from the oxidative damage (Saito *et al.*, 2003). Selenium has been shown to be an important supplement in serum- and/or protein-free media for CHO (Huang *et al.*, 2007; Zhang *et al.*, 2006), Jurkat (Saito *et al.*, 2003), hybridoma (Eto *et al.*, 1991) and NS0 (Spens and Haggstrom, 2007) cell lines. Therefore, these three components appeared to have the potential for replacing some of the functions of serum in cultivation of hybridoma 192. Unfortunately, they were not able to totally substitute for all the functions of the serum in relation to hybridoma 192 and serum had to be added to the medium at a reduced level of 0.4%. This new medium afforded a growth rate that was only slightly lower than in the control medium but enhanced the average MAb production rate.

The need for a low level of serum in the redesigned medium may have been partly due to the way the statistically designed experiments were conducted. The experiment should have been run by spinning down the inoculum and washing it with phosphate buffer saline before re-suspending in the test medium. This step would have assured that no serum was carried over to the test medium via the inoculum. Unfortunately, this step was omitted unconsciously and therefore all experimental runs contained around 0.4% serum. It may be possible therefore to totally eliminate serum in a future study of hybridoma 192.

Whenever there is a change in the physical environment or the composition of the medium used in cultivating mammalian cells, there is the risk that the structure of the product protein may be affected. In view of this, the MAb produced by the cells grown in the LSD medium was examined for the possible structural changes or inactivation. Since the MAb is used for diagnostic purposes, its specificity to the antigen and the possible cross-reactivity with structurally similar hormones to the antigen, were tested (refer Section 3.6).

Figure 4.2.5 illustrates the results of the specificity and cross-reactivity assays using four steroid hormones that are structurally similar to 17-α-hydroxyprogesterone (17OHP), the antigen of the antibody. Only 5-pregnen-3β-ol-20-one (PREG5), which has a very close structural similarity to 17OHP, had a slight cross-reactivity with the antibody at a high concentration of PREG5. No cross-reactivity was observed for the other hormones. Figure 4.2.5 further shows that the MAb produced in the LSD media had a high specificity to its antigen, 17OHP. Thus, substituting the serum in the medium, or the new formulation of the medium, did not affect the activity of the MAb produced.



Key

17OHP	17α-hydroxyprogesterone
PREG17	17α-hydroxypregnenolone
PREG5	5-pregnen-3β-ol-20-one
ANDRD	4-androstene-3,17-dione
BESTR	β-estradiol

Figure 4.2.5: MAb specificity and cross-reactivity with steroid hormones with structures similar to 17OHP

4.2.5 Conclusion

The culture medium for the hybridoma 192 was successfully optimized by using statistical experimental design. Serum content in the medium was reduced to 0.4% while requiring only inexpensive supplements, i.e. 311.8 µM ferric citrate, 4.5 µM zinc sulfate and 17.3 nM sodium selenite. With this new medium or LSD, the cost of the medium was reduced by as much as 70%. The specific growth rate of cells in the LSD was slightly lower as compared to that in 2% DMEM, while the average specific MAb production rate and the MAb titer were greatly enhanced. The activity of the MAb produced was not affected by the new medium formulation.

4.3 Screening and Optimization of Inducer Agents

4.3.1 Introduction

In view of the low specific MAb production rate (0.0569 ± 0.0453 pg/cell.h) obtained (Section 4.2.4.4) as compared to the values reported by other researchers that used other types of hybridoma cells (e.g. 1.0 pg/cell.h by Bloemkolk *et al.* (1992) using hybridoma S4B6 in an agitated serum-containing culture), a method needed to be found to stimulate the MAb production. One possible way was to add an inducer agent to the culture. The possible inducer agents identified through literature review were essential amino acids, lysozyme, lipopolysaccharides (LPS) and dimethyl sulfoxide (DMSO).

4.3.2 Objectives of study

The objective of the study was to screen and optimize the inducer agents that could increase the cell specific MAb productivity and MAb titer of the cells cultured in the LSD medium. The target was to achieve a minimum of 2-fold increase in specific MAb productivity and MAb titer.

4.3.3 Materials and methods

4.3.3.1 Chemicals

DMSO, LPS (from *E. coli*, O26:B6), lysozyme (from chicken egg white), MEM essential amino acids solutions and other chemicals were all obtained from Sigma-Aldrich.

4.3.3.2 Screening of inducer agents

The four components specified above (Section 4.3.3.1) were screened for their effectiveness in inducing the production of MAb in the following concentration range: amino acids solution (0 – 5% v/v), lysozyme (0 – 500 µg/mL), LPS (0 – 25 µg/mL), DMSO (0 – 5% v/v). The only response that was monitored was the MAb titer attained at the time of harvest (i.e. t = 72 h).

4.3.3.3 Cell culture condition during inducer screening

Cells were thawed as usual into a T75 flask and subcultured twice in the LSD before the experiments. When cell density had reached a sufficient quantity and cell viability had exceeded 90%, 2×10^5 cells/mL were spun and re-suspended in the test medium (i.e. LSD with the specified amount of a specified inducer present). It was then re-distributed in a 6-wellplate in triplicate wells per test medium. The total culture volume was 2 mL per well. All cultures were kept in the humidified CO_2 incubator with 5% CO_2 at 37°C. Samples were harvested at 72 h, spun (500*g*, 10 min) and the supernatant was kept at -20°C until further analysis. MAb titer was analyzed as stated in Chapter 3.

4.3.3.4 Optimization of inducer agent

Based on screening experiments with the above mentioned 4 inducers, only LPS was selected for a further detailed study. Optimum concentration of LPS was determined using the response surface methodology. One factor central composite experimental design was chosen and the experiment was designed using Design Expert version 7.0. The LPS concentration range chosen for optimization was based on the results of screening (8 – 12 µg/mL). There were a total of 7 runs as depicted in Table 4.3.1. The responses that were monitored were the MAb titer attained at 72 h and the average specific MAb production rate. Data obtained from duplicate independent runs were used for the analysis.

Table 4.3.1: LPS concentration in the experimental runs of the one factor central composite design

Run	LPS Concentration (µg/mL)
1	12
2	8
3	9
4	12
5	11
6	10
7	8

4.3.3.5 Cell culture condition during optimization process

Similar to the screening process, 2×10^5 cells/mL were spun and re-suspended in the test medium with different concentrations of LPS. T-25 flasks were used with the total volume of 10 mL. All cultures were kept in humidified CO_2 incubator with 5% CO_2 at 37°C. A 1 mL sample was taken at 0, 24, 48 and 72 h. Cell count was done immediately after sampling and the supernatant was kept at -20°C until further analysis.

4.3.3.6 Verification of inducing effect

The verification run was analogous to the optimization process runs. Cells were spun and re-suspended in the LSD medium containing 8 µg/mL of LPS or the control medium (LSD medium alone) at 2×10^5 cells/mL. Experiment was run in duplicate at 37°C in a humidified CO_2 incubator for 72 h. Sampling was done at 24 h intervals. The volume of each sample was 1 mL.

4.3.4 Results and discussion

4.3.4.1 Screening of significant factors

The MAb titer obtained in the presence of various concentrations of the inducer agents is shown in Figure 4.3.1, Figure 4.3.2, Figure 4.3.3 and Figure 4.3.4 for DMSO, lysozyme, amino acids, and LPS, respectively. The MAb titer at each concentration for a specific factor was the average of triplicate wells, and the results plotted were the average value of duplicate analyses.

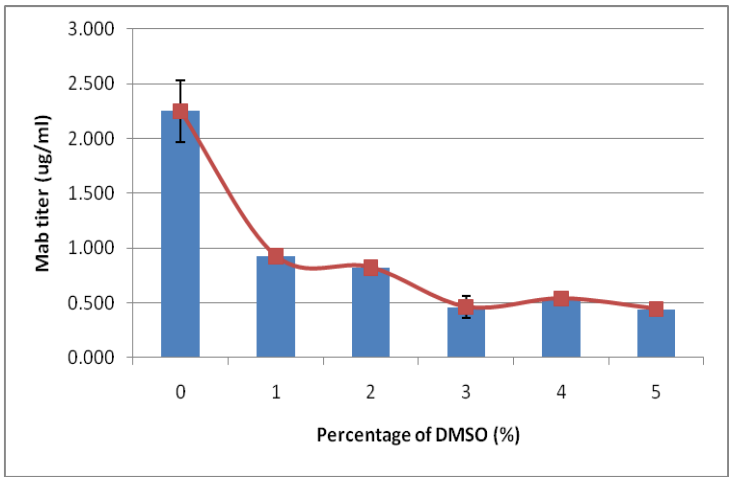


Figure 4.3.1: MAb titer at 72 h in media supplemented (after spinning) with various concentrations DMSO (%v/v)

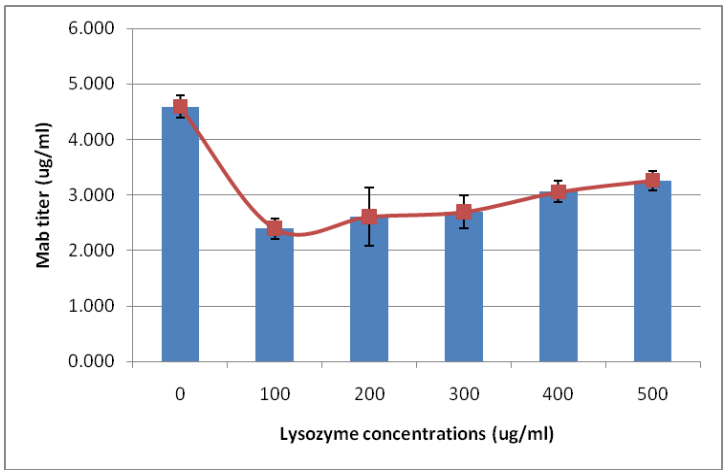


Figure 4.3.2: MAb titer at 72 h in media supplemented with various lysozyme (µg/mL) concentrations

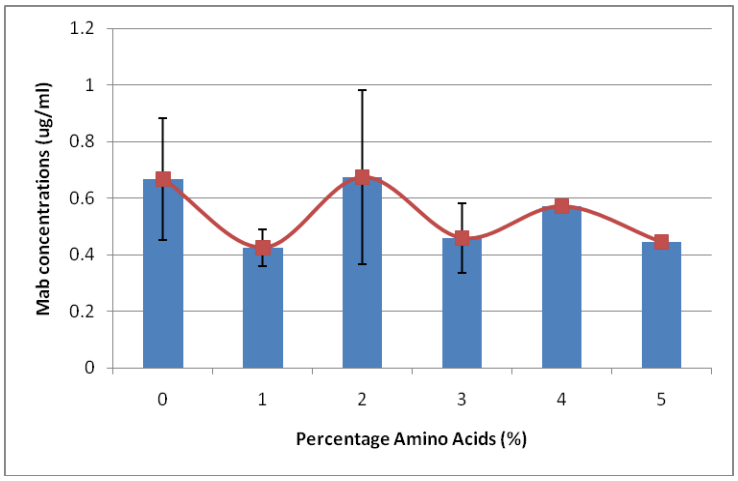


Figure 4.3.3: MAb titer at 72 h in media supplemented with amino acids solution at various concentrations (%v/v)

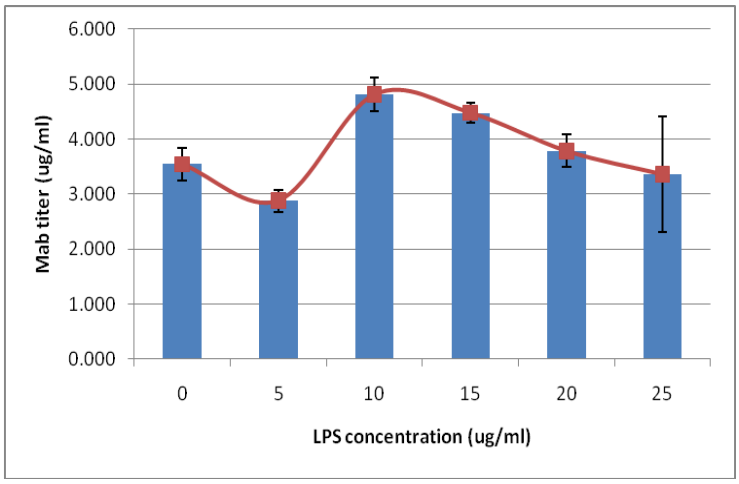


Figure 4.3.4: MAb titer at 72 h in media with various cocentrations of LPS (µg/ml)

In view of Figure 4.3.1, Figure 4.3.2 and Figure 4.3.3, the presence of DMSO, lysozyme and essential amino acids in the medium did not produce any significant inducing effect on MAb. In fact, addition of DMSO and lysozyme reduced the MAb production. The MAb titer at the time of harvesting was very much lower in DMSO-containing media as compared to the control. The MAb titer was about 60% of that in the control for the medium which contained lysozyme. Amino acids, on the other hand, did not show any positive or negative influence on the MAb production. The results obtained in this study are in contrast to the earlier findings in relation to DMSO (Ling *et*

al., 2003; Wang *et al.*, 2007), lysozyme (Sugahara *et al.*, 2005; Murakami *et al.*, 1997), and amino acids (Ducommun *et al.*, 2001; Gong *et al.*, 2006).

By feeding amino acids solution, Gong *et al.* (2006) were able to increase the MAb production of JJ-1 cells from 116 mg/L up to 707 mg/L. A similar observation was reported by Ducommun *et al.* (2001), where balanced feeding of amino acids and vitamins had increased the specific IgA production of Zac 3 cells from 0.60 pg/cell.h to 1.10 pg/cell.h. However, addition of essential amino acids in this study did not show any enhancement of MAb production. A possible reason is that the other researchers specially formulated the amino acids feed according to the consumption requirements of cells, but in this study the general essential amino acids solution was added at the beginning of the culture without considering the consumption by the cells. Franěk *et al.* (2003) reported that addition of free amino acids (glycine/lysine mixture) did not enhance MAb production, but the addition of Gly-Lys-Gly tripeptide did. Thus, the enhancing effect of amino acids are specific to the cells, highly dependent on the type of amino acids being fed and also the time of feeding.

Murakami *et al.* (1997) found that 380 µg/mL of lysozyme was able to stimulate the production of IgM up to 13-fold by HB4C5 hybridoma cells. The finding in this research, however, was that lysozyme at the range of 0 - 500 µg/mL had no stimulatory effect on MAb production by hybridoma 192 cells. Therefore, the effect of lysozyme might be cell specific.

Ling *et al.* (2003) reported that the productivity of their hybridoma culture increased 2-fold following the addition of 0.2% (v/v) DMSO at the time of the maximal viable cell densities. A 3-fold increase in productivity of hepatitis B surface antigen by CHO cells when supplemented with 1.5% (v/v) DMSO at 96 h of culture has been reported (Wang *et al.*, 2007). The present work studied the stimulating effect of DMSO in the concentration range of 0 – 5% (v/v). Two independent sets of experiments were run. The first duplicate set of experiments was run without spinning the cells prior to re-suspending into the fresh test medium that contained 0 – 2.5% (v/v) of DMSO. The results are depicted in Figure 4.3.5. Due to carry over of the spent medium in the inoculum, the real concentration of DMSO in the study was less than the values shown in Figure 4.3.5. When the error was realized, the experiment was repeated with a

broader concentration range of DMSO and the cells were spun prior to re-suspending. The results were as shown in Figure 4.3.1. Figure 4.3.1 and Figure 4.3.5 display a totally different behavior. In the experiment with low concentration of DMSO and carryover of spent medium (Figure 4.3.5), there was a significant increase in MAb titer at the time of harvesting (i.e. 72 h). In addition, MAb productivity was found to increase in this study. Cells tended to lyse and growth was suppressed when DMSO was added to the culture as previously reported by others (Ling *et al.*, 2003; Wang *et al.*, 2007). Lysed cells released the antibody that may not have fully formed, into the culture (Juanola *et al.*, 2009). However, in Figure 4.3.1 where the cells were spun and the DMSO concentration studied was broader in range, the MAb titer declined with increased concentration of DMSO. Thus, the response of cells toward DMSO was somewhat inconsistent. This was in agreement with the findings of Allen *et al.* (2008), who found that the effect of DMSO on the cells could only be seen at the concentration above 1% (v/v), but this effect was not consistent. Since the stimulatory effect of DMSO could not be assured, while amino acids produced no benefit and lysozyme had a negative effect, DMSO, amino acids and lysozyme were excluded from further study.

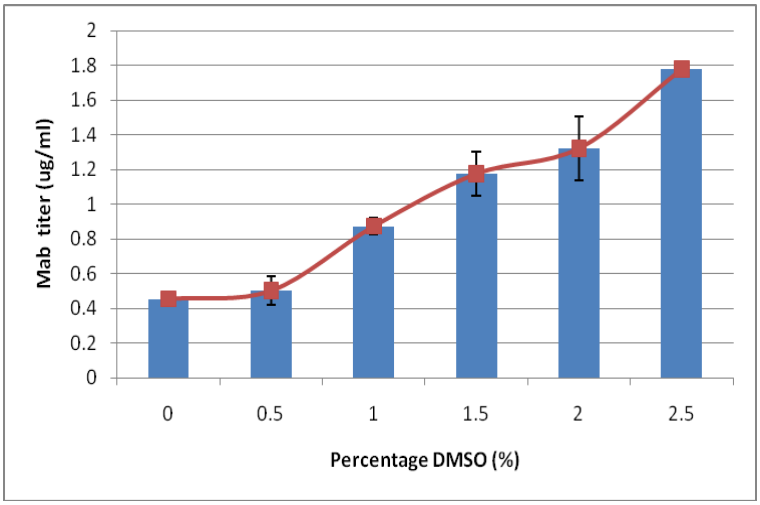


Figure 4.3.5: MAb titer at 72 h in media supplemented with various concentrations of DMSO (%v/v) (without spinning)

Figure 4.3.4 shows the MAb titer attained at 72 h with the various concentrations of LPS in the medium. At LPS concentration between 5 – 15 µg/mL, there was a significant increase in MAb titer as compared to the control (LPS

concentration = 0 µg/mL). Since a preliminary earlier study using LPS at a similar concentration range also enhanced the MAb titer as compared to control, the stimulation effect of LPS seemed quite promising. As a result, LPS was further optimized in the above noted concentration range.

4.3.4.2 Determination of optimum LPS concentration

Table 4.3.2 illustrates the results of ANOVA for optimization using the central composite design. It reveals that increasing LPS concentration will have a negative effect on the maximum MAb titer and specific MAb production rate. In other words, increased LPS concentration would decrease the maximum MAb titer attained and reduce the specific MAb production rate. The significant term in both models is the quadratic term, A^2. LPS concentration is also significant in the average specific MAb production rate model.

Table 4.3.2: Analysis of variance table for maximum MAb titer and specific MAb production rate

Factors	Maximum MAb titer			Average specific MAb production rate		
	Effect	F-ratio	p-value	Effect	F-ratio	p-value
A – LPS concentration	-0.38	2.32	0.2020	- 0.008	8.37	0.0444*
A^2	1.69	12.36	0.0246*	0.048	82.46	0.0008*
Lack of Fit	-	0.49	0.6693	-	3.18	0.2391

* Significant at $p < 0.05$

The experimental results obtained were fitted to the quadratic model equations in coded form as follows:

$$Maximum\ MAb\ titer = 6.25 - 0.38\ A + 1.69\ A^2 \quad \text{(Eq.4.3.1)}$$

$$Specific\ MAb\ production\ rate = 0.11 - 0.008\ A + 0.048\ A^2 \quad \text{(Eq.4.3.2)}$$

Table 4.3.3 shows the goodness of fit (R^2) and the goodness of prediction (Q^2) for the models. R^2 for average specific MAb production rate was greater than 0.75, revealing that the model did fit well, whereas the model for the maximum MAb titer only could be considered a fair model as the R^2 was less than 0.75. The Q^2 value for

maximum MAb titer was above 0.25 and below 0.75, while the Q^2 for average specific MAb production rate was well above 0.75. Therefore, the models were expected to be able to predict the optimum values reasonably well. Based on the models above, the optimum point was predicted to be at an LPS concentration of 8 µg/mL. At this optimum point the maximum MAb titer and the specific MAb production rate were predicted to be 8.318 µg/mL and 0.1614 pg/cell.h, respectively. This optimum point was verified by running a duplicate experiment at the predicted optimal LPS concentration of 8 µg/mL. A control was also run in duplicate for comparison.

Table 4.3.3: Goodness of fit (R^2) and goodness of prediction (Q^2) for the models

Responses	**R^2**	**Q^2**
Maximum MAb titer (Eq. 4.3.1)	0.6788	0.3215
Average specific MAb production rate (Eq. 4.3.2)	0.9367	0.8791

4.3.4.3 Verification of the optimum point

Table 4.3.4 compares the actual experimental values of the maximum MAb titer and the average specific MAb production rate with the predicted values and the control values. It can be seen that the actual maximum MAb titer obtained is lower than both the predicted and control values. This is obviously in contrast to the results obtained during screening and optimization process. The reason behind this is not clear and needs further investigation. Yet, Table 4.3.4 also reveals that the actual average specific MAb production rate obtained is very much higher than both the predicted and control values. The increment of productivity is up to 61% (or 1.6-fold) as compared to the control. Addition of LPS into the culture medium had a suppressory effect on growth as did DMSO (Ling *et al.*, 2003; Wang *et al.*, 2007; Martin-Lopez *et al.*, 2007). However, LPS did not lyse the cells. As observed in this study, LPS only suppressed the growth of cells for the first 24 to 48 hours. Cell grew as usual afterwards. This suppressory effect reduced the cell number at the time of harvesting. Since the average specific MAb production rate was calculated based on the maximum MAb titer achieved using the viable cell density attained at the instance of maximum MAb titer, the average specific MAb production rate value was elevated.

Table 4.3.4: Comparison of the predicted, actual and control values

Type of response	Maximum MAb titer (µg/mL)	Average specific MAb production rate (pg/cell.h)
Predicted	8.318	0.1614
Actual	6.181 ± 0.348	0.2532 ± 0.0167
Control	7.304 ± 0.839	0.1577 ± 0.0071

Even though LPS suppressed growth, its performance was inconsistent. The suppression effect was not observed in the first optimization experiment, but was obvious in the second optimization experiment and also in the verification run. This may be the reason that maximum MAb titers obtained were higher in the first optimization experiment, but not in the second optimization experiment or the verification run. Nonetheless, both the predicted and the actual average specific MAb production rate were higher compared to the control (Table 4.3.4). The following could be a possible reason for the lower maximum MAb titer at the time of harvest as compared to the control in verification run (Table 4.3.4): At the time of harvesting (i.e. 72 h), cells supplemented with LPS had just returned to their normal growth rate and the cell number was lower than the control culture. As a result, the MAb titer produced was also lower than the control culture that contained a higher cell number. If the culture was harvested at a later time, the maximum MAb titer achieved might have improved. LPS is believed to be able to stimulate immune response of mammalian cells, but suppresses growth (Martin-Lopez *et al.*, 2007; Oliver *et al.*, 1999). Thus, this could be a plausible explanation for the enhancement of MAb production in this case.

Martin-Lopez *et al.* (2007) found that by adding 500 pg/mL of LPS to culture of 55-6 murine B cell hybridoma, the MAb production rate could be increased by 2.5-fold as compared to the control. In this study, the LPS concentration used was 8 µg/mL, which was 16,000-fold greater than that used by Martin-Lopez *et al.* (2007). Nevertheless, the enhancement of MAb production was only 1.6-fold. Different cells may differ in their responses to the LPS at different concentrations (Oliver *et al.*, 1999). For example, Oliver *et al.* (1999) observed that the ability of marginal zone B cells and follicular B cells to proliferate and differentiate into plasma cells dependend on the LPS concentration added.

LPS is expensive. Table 4.3.5 shows the cost of the medium if LPS is added to it at a concentration of 8 µg/mL. Detailed calculations are shown in Appendix C (page 209). Adding LPS will increase the cost of the medium by 40-fold compared to the LSD medium. Thus, the addition of LPS, which enhanced MAb production by only 1.6-fold, is not practicable.

Table 4.3.5: Comparison of media costs with and without LPS

Type of medium	Cost per L (RM)
LSD	39.03
LSD supplemented with LPS	1,559.03

4.3.5 Conclusion

LPS at a concentration of 8 µg/ml was found to be suitable for stimulating the MAb production. The verification process confirmed that LPS was able to enhance the average specific MAb production rate by 1.6-fold relative to control, but not the maximum MAb titer. As the performance of LPS was inconsistent and its ability to induce MAb production was low compared to its cost, it was not investigated further. All future experiments were carried out without any LPS, that is in LSD medium which consisted of DMEM supplemented with 0.4% FBS, 4 mM L-glutamine, 1% antibiotic-antimycotic, 0.1% Pluronic F68, 311.8 µM ferric citrate, 4.5 µM zinc sulfate and 17.3 nM sodium selenite.

4.4 Operational Parameter Optimization Using Statistical Experimental Design

4.4.1 Introduction

Production of monoclonal antibody under controlled conditions is necessary to assure a consistent quality for clinical diagnostic use. A stirred tank bioreactor configuration has been selected here for process scale up as it is the most commonly used type of fully controlled bioreactor in the industry. Hybridoma 192 was previously cultured under partially controlled conditions in static T-flasks, where the pH was only roughly regulated. Also, the oxygen level was not controlled. As the susceptibility of the cells to shear generated by sparging and stirring were unknown, the various operating parameters in the fully-controlled bioreactor needed to be determined and optimized for optimum production of monoclonal antibody. A preliminary study was run in spinner flasks prior to the screening and optimization of the operating parameters in a 2L bioreactor. This was done to obtain some idea of the oxygen requirements and the susceptibility of the cells to shear. This information was expected to aid the subsequent work in the bioreactor.

4.4.2 Objectives of study

The objective of the study was to screen and optimize the operating parameters in a 2 L bioreactor to obtain a comparable cell growth and production of MAb in the LSD medium as in the T-flask cultures.

4.4.3 Materials and methods

4.4.3.1 Preliminary study in spinner flask culture

Cell growth profiles in LSD medium were obtained by culturing in different sizes of spinner flasks at different stirring speeds. Cells from mid-log phase with a viability greater than 90% were inoculated at an inoculum density of 2×10^5 cells/mL into either 250 mL or 100 mL spinner flasks, at a final total volume of 90 mL. The culture was stirred at a speed of either 40 rpm (250 mL and 100 mL flasks) or 75 rpm

(100 mL flask) and kept in the humidified CO_2 incubator at 37°C. A 1.8 mL sample was taken after 24 h and twice daily subsequently until the viability dropped to or below 30%.

4.4.3.2 Experimental design for the screening process

A two-level factorial design was used in screening the significant operating parameters. A 2^{5-1}_{V} design was selected with 3 center points and thus a total of 19 runs. Five factors that were screened were temperature (33 - 37°C), pH (6.8 – 7.4), dissolved oxygen (30 – 70%), stirring speed (70 – 100 rpm) and sparging rate (0.02 – 0.10 vvm). A total of 4 responses were monitored, they were the maximum viable cell density, the specific growth rate, the maximum MAb titer and the average specific MAb production rate.

4.4.3.3 Cell culture procedures

Cells were thawed as usual into a T75 flask (Nunc, Denmark) and subcultured once before inoculating into the spinner flask (Cellspin, Integra Bioscience). When cell density had reached a sufficient quantity and cell viability had exceeded 90%, 1.5 – 2.5 $\times$ 10^5 cells/mL were inoculated into LSD medium in a 2L bioreactor (Biotron, Korea). The total culture volume was 1.25 L. 25 mL samples were taken at 0 h and 2 – 3 times daily in the subsequent days until the cell viability had fallen to less than 30%. Cell count and MAb titer were analyzed as described in Chapter 3.

4.4.3.4 Augmentation to central composite design

Two-level fractional factorial design was augmented to central composite design by using Design Expert version 7. Faced centered design was chosen. An extra 12 runs were added to the design, where 2 of them were center points. Each factor was varied between the minimum and maximum (see Section 4.4.3.2), one at a time, in which all other factors remained at their center point values. Culture procedure was exactly the same as described for the screening experiment (Section 4.4.3.3).

4.4.3.5 Verification of optimum point

The optimum point was obtained from the ANOVA analysis (Design Expert version 7). This point was verified by running a verification experiment in triplicate at the calculated optimum setting provided by the software. The culture procedure was similar to that described for the screening experiments (Section 4.4.3.3).

4.4.4 Results and discussion

4.4.4.1 Preliminary study in spinner flask culture

Figure 4.4.1 shows the typical growth curves of the cells in static T-flask and stirred spinner flask cultures. The maximum viable cell density achieved in spinner culture was higher than in the T-flask culture (1.80×10^6 cells/mL and 1.50×10^6 cells/mL, respectively). The cell growth rate was apparently slightly higher in the spinner flask culture. Table 4.4.1 compares the specific growth rate and the average specific MAb production rate of the cells in T-flask cultures and spinner flask cultures. The results were averages of quadruplicate runs. Table 4.4.2 provides the surface-to-volume ratio of 100 mL and 250 mL spinner flasks at the culture volume used. A high surface-to-volume ratio means that more surface area is available to the culture for absorption of oxygen.

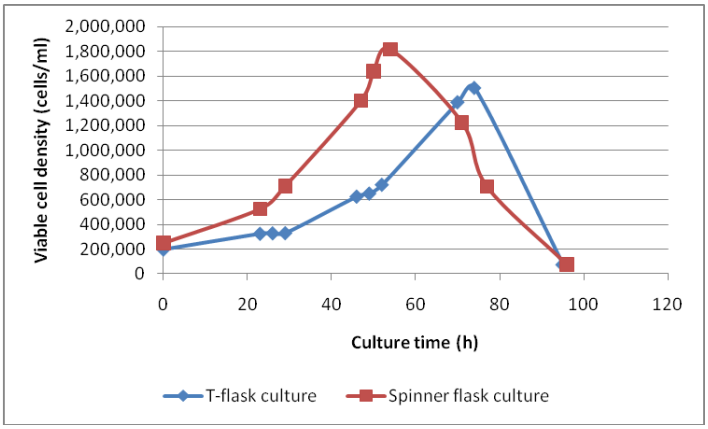


Figure 4.4.1: Comparison of growth curves for cells in T-flask and spinner flask cultures (100 mL flask, 40 rpm)

Table 4.4.1: Comparison of specific growth rates and average specific MAb production rate of hybridoma 192 in T-flask and spinner flask cultures

Culture system	Specific growth rate (1/h)	Average specific MAb production rate (pg/cell.h)
T-flask	0.033 ± 0.001	0.0569 ± 0.0453
Spinner flask (100 mL flask, 40 rpm)	0.038 ± 0.002	0.1436 ± 0.0451
Spinner flask (100 mL flask, 75 rpm)	0.045 ± 0.002	0.0994 ± 0.0674
Spinner flask (250 mL flask, 40 rpm)	0.034 ± 0.001	0.0597 ± 0.0029

Table 4.4.2: The surface-to-volume ratio of spinner flasks for a medium volume of 90 mL

Size of flask	Surface-to-volume ratio, cm^{-1}
100 mL flask	0.345
250 mL flask	0.526

A 250 mL size spinner flask has a higher surface-to-volume ratio compared to a 100 mL flask (Table 4.4.2). A higher ratio means a reduced difficulty in oxygen transfer. It also means that oxygen can be transferred easily from the surface of the medium to the bottom of the flask both through surface aeration and with the aid of the stirrer. In a relatively deep culture not sparged with air, a high surface-to-volume ratio is expected to translate into a higher specific growth rate. Surprisingly, enhancing oxygen transfer to the cells did not seem to improve the specific growth rate as expected. As illustrated in Table 4.4.1, the specific growth rate of the cells in the 250 mL size flask is comparable to that in the 100 mL flask at 40 rpm. This result was contrary to that obtained in Section 4.1.4.4 for cells growing in DMEM supplemented with 2% or 5%

serum, where the specific growth rate increased when the surface-to-volume ratio increased.

Stirring speed, on the other hand, appeared to affect the specific growth rate (Table 4.4.1). There was an enhancement of specific growth rate at a higher speed in the same size of flask (100 mL; Table 4.4.1). Higher stirring speed encourages better oxygen transfer to the medium from the surface. Stirring also helps mix and suspends the cells, which in turn exposes all the cell surfaces for oxygen and nutrient transfer. Nevertheless, stirring also introduces shear to the cells. Oxygen was believed to be a limiting factor in T-flask culture of hybridoma 192. Tendency of the hybridoma cells to deposit at the base of the T-flask limited the exposure of the cells' surface to nutrients and oxygen. Thus, improved mixing and oxygen transfer while limiting the stirring speed, so that the shear effects were low, enhanced the specific growth rate. Since the results were consistent with the theory, it could be concluded that the speeds used in this 100 mL flask with a single pendulum agitator (Figure B1, Appendix B) was not imposing a significant shear effect on the cells. However, the 250 mL flask with its dual pendulums (Figure B2, Appendix B) may have imposed more shear on the cells at the same stirring speed as the single pendulum. This negative effect was counteracted by the positive effect of a better oxygen transfer. Thus, no improvement in specific growth rate was observed in the LSD medium. This finding consistent with those in Section 4.1.4.4, where the shear effect become obvious in 250 mL size spinner flask when the stirring speed was increased to 75 rpm in the medium supplemented with a higher serum content. LSD medium contained less serum and thus the shear effect was more apparent here.

Table 4.4.1 also compares the average specific MAb production rate for the different culture scenarios. The best rate was obtained in the 100 mL size spinner culture stirred at 40 rpm, following by the culture grown in the same size of spinner but at 75 rpm stirring speed. The average specific MAb production rate of cells cultured in the 250 mL size spinner flask and stirring at 40 rpm was comparable to the ones cultured in the T-flask. Under stress condition, such as existence of shear, animal cells tend to produce more product (Miller *et al.*, 1988a). Therefore, it was expected that a higher productivity could be obtained in the spinner flask culture. However, shear effect of stirring lysed the cells and reduced productivity in the 250 mL size of spinner culture.

A layer of cell debris was typically observed at the walls of the flask in this culture. In 100 mL size spinner culture, stirring had improved oxygen transfer at 75 rpm, and this increased the specific growth rate despite a higher shear force. Owing to the negatively growth associated characteristic of MAb, the average MAb production rate did not increase. At 40 rpm, the 100 mL size spinner culture showed only slight increase in the specific growth rate compared to the T-flask culture, but a greater improvement in the average MAb production rate. This may be due to the stirring being introduced here being only sufficient to suspend and mix the cells, but not sufficient to boost the growth rate or cause cell lysis.

From this preliminary study, it can be seen that both the specific growth rate and the average MAb production rate are dependent on the stirring speed and dissolved oxygen in the medium through a complicated interaction. Shear effect does play a role in affecting both the growth and production of hybridoma 192. To ensure a successful scale up and optimization into the 2 L fully controlled bioreactor, a screening and optimization process should be gone through with the aid of statistical experimental design.

4.4.4.2 Screening of significant factors

Table 4.4.3 shows the analysis of variance (ANOVA) for the maximum viable cell density and the specific growth rate, while Table 4.4.4 shows the ANOVA for the maximum MAb titer and the average specific MAb production rate in the screening study. All five factors significantly influenced the maximum viable cell density attained by hybridoma 192 in the bioreactor (Table 4.4.3). Specific growth rate, on the other hand, was significantly affected only by the temperature. Both responses showed a significant positive curvature, which meant that the optimum point was within the range of the variables used in the study. As shown in Table 4.4.4, stirring speed, pH and temperature were the significant factors that affected the maximum MAb titer. No significant curvature was detected in this response. In contrast, the average specific MAb production rate was significantly affected by aeration rate, pH and temperature. This response had a significant negative curvature.

Table 4.4.3: Analysis of variance table for the maximum viable cell density and the specific growth rate in screening study

Factors	Max viable cell density			Specific growth rate		
	Effect	**F-ratio**	**p-value**	**Effect**	**F-ratio**	**p-value**
A – Aeration rate	0.00015	114.04	0.0018*	0.062	1.07	0.3769
B – Stirring speed	-0.00007	24.38	0.0159*	0.13	4.78	0.1166
C – pH	0.00007	26.69	0.0141*	0.043	0.50	0.5309
D – Temperature	-0.00010	47.43	0.0063*	-0.32	27.97	0.0132*
E – Dissolved oxygen	0.00006	16.18	0.0276*	-0.13	4.30	0.1298
AB	-0.00014	106.94	0.0019*	0.070	1.34	0.3308
AC	-	-	-	-0.21	12.15	0.0399*
AD	-0.00008	37.68	0.0087*	-0.076	1.61	0.2943
AE	0.00010	48.04	0.0062*	-0.20	10.72	0.0466*
BC	-0.00011	59.06	0.0046*	-0.18	8.85	0.0588
BD	0.00006	20.66	0.0199*	0.23	14.97	0.0305*
BE	-0.00008	35.38	0.0095*	-	-	-
CD	0.00001	1.15	0.3615	0.092	2.31	0.2262
CE	0.00018	168.21	0.0010*	0.29	23.73	0.0165*
DE	0.00004	10.47	0.0480*	0.38	38.92	0.0083*
Curvature		73.87	0.0033*		21.65	0.0187*
Lack of Fit		0.047	0.8487		0.055	0.8358

* Significant at $p < 0.05$

Table 4.4.4: Analysis of variance table for the maximum MAb titer and the average specific MAb production rate in screening study

Factors	Max MAb titer			Average specific MAb production rate		
	Effect	**F-ratio**	**p-value**	**Effect**	**F-ratio**	**p-value**
A – Aeration rate	0.045	0.026	0.8779	0.015	11.92	0.0062*
B – Stirring speed	0.98	11.97	0.0135*	0.0025	0.36	0.5632
C – pH	1.72	37.17	0.0009*	0.054	160.52	<0.0001*
D – Temperature	1.33	22.25	0.0033*	0.028	44.34	<0.0001*
E – Dissolved oxygen	-0.67	5.55	0.0565	0.0021	0.24	0.6336
AB	0.39	1.92	0.2149	-	-	-
AC	-	-	-	-	-	-
AD	-	-	-	-	-	-
AE	-1.53	29.31	0.0016*	-0.024	32.79	0.0002*
BC	-0.80	8.05	0.0297*	-0.023	30.79	0.0002*
BD	-	-	-	-	-	-
BE	0.60	4.55	0.0770	-	-	-
CD	-1.24	19.27	0.0046*	-	-	-
CE	-0.72	6.54	0.0430*	-	-	-
DE	-	-	-	-	-	-
Curvature		1.52	0.2632		13.26	0.0045*
Lack of Fit		0.14	0.9496		0.93	0.6151

* Significant at $p < 0.05$

In determining the optimum operating conditions in the bioreactor, the maximum MAb titer and the average specific MAb production rates were considered to be the more important responses as compared to the maximum viable cell density and the specific growth rate. Even though the specific growth rate response gave the desired positive curvature, the average specific MAb production rate showed a negative curvature, which was undesired. Nonetheless, all the studied factors were found to have significant interactive effects on all the responses studied. Since the contributors to the curvature were unknown, none of the factors were discarded. The whole design was augmented to a face centered central composite design. Face centered central composite design was used instead of the orthogonal, spherical or rotatable designs because if the alpha point was more than 1, the operating parameters such as pH and temperature will be out of the normal physiological range for the cells. This was unacceptable as the cell growth and MAb production would have been inhibited. Thus, a face centered design that ensured that the operating parameters remained within the physiological range for the cells was selected.

4.4.4.3 Augmentation to central composite design

ANOVA for the maximum viable cell density and the specific growth rate after augmentation to face-centered central composite design is shown in Table 4.4.5. The ANOVA for the maximum MAb titer and the average specific MAb production rate is shown in Table 4.4.6.

Table 4.4.5: Analysis of variance table for the maximum viable cell density and the specific growth rate in central composite desgin

Factors	Max viable cell density			Specific growth rate		
	Effect	F-ratio	p-value	Effect	F-ratio	p-value
A – Aeration rate	-188700	4.73	0.0439*	-	-	-
B – Stirring speed	-44282.39	0.26	0.6162	-0.0022	3.15	0.0903
C – pH	-193800	4.99	0.0392*	-0.0016	1.76	0.1983
D – Temperature	1504.61	0.0003	0.9864	0.0034	7.75	0.0111*
E – Dissolved oxygen	-1273.17	0.0002	0.9885	0.0004	0.088	0.7696
AB	226000	6.03	0.0251*	-	-	-
AC	167400	3.31	0.0865	-	-	-
AE	-140000	2.32	0.1464	-	-	-
BC	198600	4.66	0.0454*	0.0024	3.40	0.0793
BD	-	-	-	-0.0027	4.31	0.0503
CE	-234600	6.50	0.0207*	-0.0028	4.60	0.0438*
DE	-206200	5.03	0.0386*	-0.0037	8.24	0.0091*
A^2	-457400	6.48	0.0209*	-	-	-
Lack of Fit		3.58	0.1608		2.56	0.2393

* Significant at $p < 0.05$

Table 4.4.6: Analysis of variance table for the maximum MAb titer and the average specific MAb production rate in central composite design

Factors	Max MAb titer			Average specific MAb production rate		
	Effect	**F-ratio**	**p-value**	**Effect**	**F-ratio**	**p-value**
A – Aeration rate	-0.10	0.024	0.8785	-	-	-
B – Stirring speed	1.14	2.97	0.1012	-	-	-
C – pH	1.48	5.03	0.0370*	0.053	11.89	0.0019*
D – Temperature	1.53	5.39	0.0315*	0.037	5.69	0.0243*
E – Dissolved oxygen	-1.17	3.15	0.0921	-	-	-
AE	-1.53	4.78	0.0415*	-	-	-
BC	-0.80	1.31	0.2661	-	-	-
CD	-1.24	3.14	0.0923	-	-	-
B^2	2.12	1.75	0.2015		-	-
D^2	-2.94	3.38	0.0819			
Lack of Fit		0.42	0.8949		1.31	0.4741

* Significant at $p < 0.05$

As shown in Table 4.4.5, aeration rate and pH had significant effects on the maximum viable cell density that could be attained, but the effects were negative, that is as the aeration rate and pH increased, the maximum viable cell density would be reduced. Both these factors interacted with the stirring speed, the dissolved oxygen content and temperature in affecting the response (Figure 4.4.2). As shown in Figure 4.4.2, to obtain a high maximum viable cell density, the operating parameters must be set at a low dissolved oxygen content, a high pH, a high temperature, a high stirring speed and a moderate aeration rate.

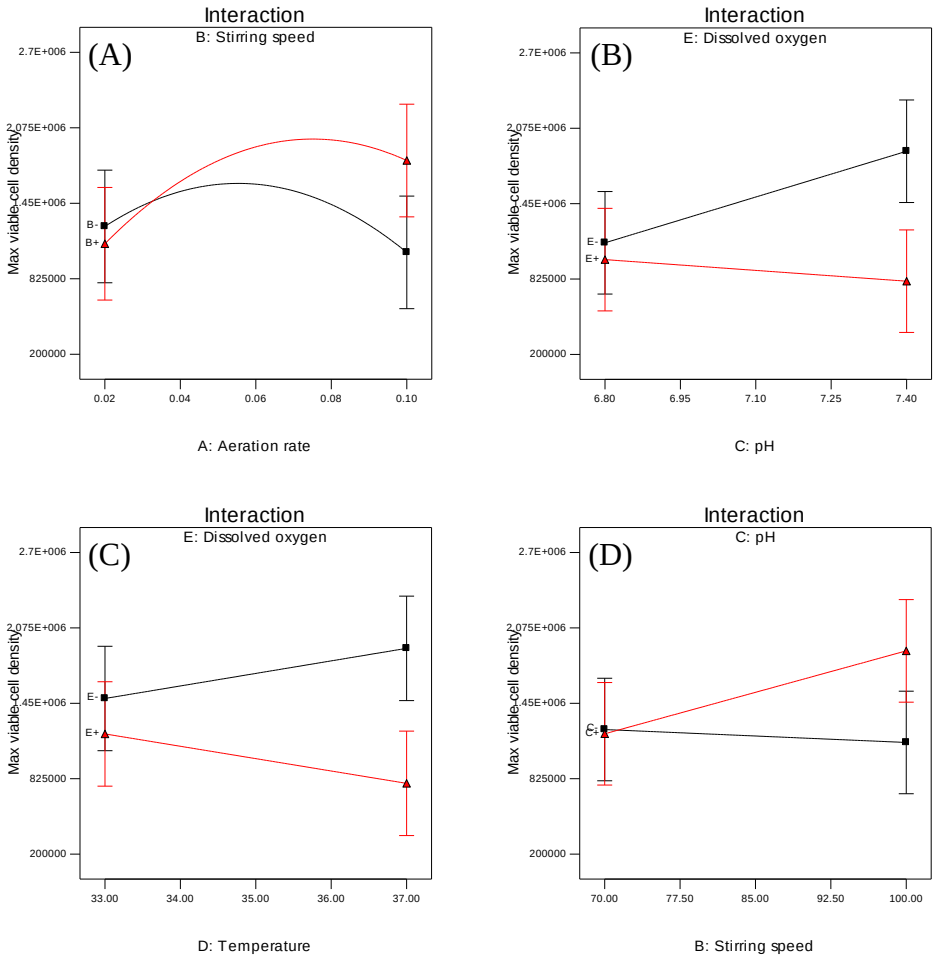


Figure 4.4.2: Significant interactions between the factors on the response of maximum viable cell density: (a) aeration rate and stirring speed; (b) pH and dissolved oxygen; (c) temperature and dissolved oxygen; and (d) stirring speed and pH. (All other factors were fixed at the optimum conditions.)

Temperature was the only significant factor affecting the specific growth rate of cells as shown in Table 4.4.5. Temperature had a significant interaction with dissolved oxygen, which in turns interacted with the pH. As temperature increased, the specific growth rate also increased. A higher specific growth rate could be achieved if dissolved oxygen content was low and the pH was high, as shown in Figure 4.4.3.

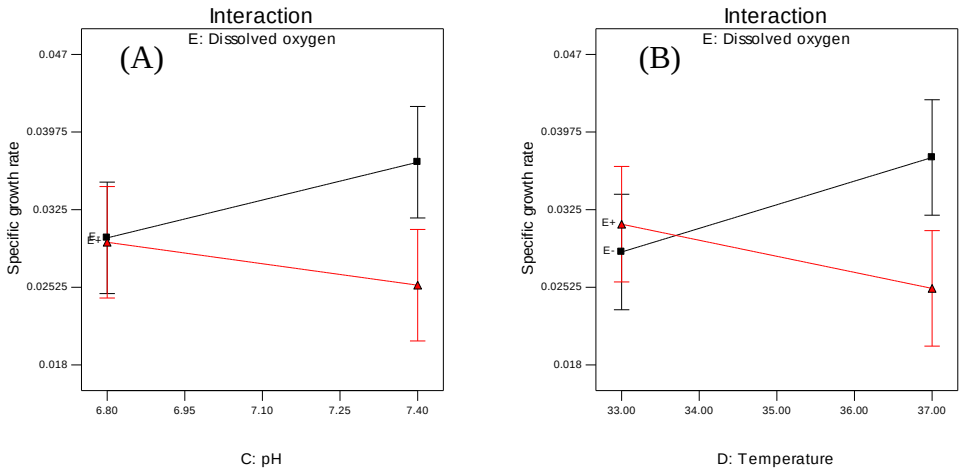


Figure 4.4.3: Significant interactions between the factors on the response of specific growth rate: (a) pH and dissolved oxygen; and (b) temperature and dissolved oxygen. (All other factors were set at the optimum conditions.)

As shown in Table 4.4.6, both maximum MAb titer and the average specific MAb production rate were significantly affected by pH and temperature. At a high temperature or pH, a high value of maximum MAb titer and the average specific MAb production rate were obtained. Interactions between aeration rate and dissolved oxygen also significantly affected the maximum MAb titer that could be achieved.

To summarize, in order to ensure a high specific growth rate and a high specific MAb production rate, together with a high maximum viable cell density and a high maximum MAb titer, the operating parameters of temperature, pH and stirring speed should be set at high levels, while the dissolved oxygen should be set at a low level with a moderate setting of the sparging rate.

The results obtained were fitted to the following model equations in coded form:

$$\textbf{\textit{Max viable cell density}} = 1.533\times 10^{6} - 1.887\times 10^{5} A - 4.428 \times 10^{4} B - 1.938 \times 10^{5} C + 1.505 \times 10^{3} D - 1.273 \times 10^{3} E + 2.260 \times 10^{5} AB + 1.674 \times 10^{5} AC - 1.400 \times 10^{5} AE + 1.986 \times 10^{5} BC - 2.346 \times 10^{5} CE - 2.062 \times 10^{5} DE - 4.574 \times 10^{5} A^{2} \quad \text{(Eq. 4.4.1)}$$

$$\textbf{Specific growth rate} = 0.032 - 2.161 \times 10^{-3} B - 1.617 \times 10^{-3} C + 3.389 \times 10^{-3} D + 3.611 \times 10^{-4} E + 2.381 \times 10^{-3} BC - 2.681 \times 10^{-3} BD - 2.769 \times 10^{-3} CE - 3.706 \times 10^{-3} DE \quad \text{(Eq. 4.4.2)}$$

$$\textbf{Max MAb titer} = 7.93 - 0.10 A + 1.14 B + 1.48 C + 1.53 D - 1.17 E - 1.53 AE - 0.80 BC - 1.24 CD + 2.12 B^2 - 2.94 D^2 \quad \text{(Eq. 4.4.3)}$$

$$\textbf{Average specific MAb production rate} = 0.13 + 0.053 C + 0.037 D \quad \text{(Eq. 4.4.4)}$$

As the average specific MAb production rate was the most important response to be optimized, followd by maximum MAb titer, the specific growth rate and maximum viable cell density (in the order of decreasing importance), the optimum operating point could be obtained from the above equations. The optimum operating parameters obtained were 0.09 vvm of aeration rate, a stirring speed of 100 rpm, a pH of 7.4, a temperature of 36.8°C and a dissolved oxygen level of 30%. The predicted value of the maximum viable cell density was 1.92 $\times 10^6$ cells/mL at a specific growth rate of 0.0368/h. The predicted maximum MAb titer was 12.07 µg/mL with an average specific MAb production rate of 0.216 pg/cell.h.

Table 4.4.7 shows the goodness of fit (R^2) and the goodness of prediction (Q^2) for all 4 models (i.e. Eq. 4.4.1 – Eq. 4.4.4). Models with an R^2 of greater than 0.75 and Q^2 of greater than 0.60 are normally considered as good (Mandenius and Brundin, 2008). Those with R^2 and Q^2 of 0.25 or less are considered useless (Mandenius and Brundin, 2008) and those in between are regarded as fair models. As shown in Table 4.4.7, all four model equations had R^2 values of greater than 0.25, but below 0.75. Thus, these models fitted the data fairly well. However, the Q^2 for all four models were less than 0.25; therefore, the predictive power for the models is quite poor. Thus, the model equations were not capable of predicting new data reasonably. Even so, the signal to noise ratios for all models were greater than the minimum of 4 (Table 4.4.7); hence, the signal was considered to be large enough to still be seen. In other words, the models were not useful for prediction purposes, but were useful for detecting the active factors. Since the models did not have good predictive power, confirmation runs needed to be

done in order to verify the predicted optimum point and to confirm that the culture does perform reasonably well at the predicted optimal point.

Table 4.4.7: Goodness of fit (R^2), goodness of prediction (Q^2) and the signal to noise ratio for the models

Responses	R^2	Q^2	Signal/noise
Max viable cell density (Eq. 4.4.1)	0.5270	0.1540	9.557
Specific growth rate (Eq. 4.4.2)	0.4661	0.0941	7.936
Max MAb titer (Eq. 4.4.3)	0.4003	0.2328	7.651
Average specific MAb production rate (Eq. 4.4.4)	0.3495	0.2385	8.414

4.4.4.4 Verification of the optimum point

The optimum point was verified by culturing the cells in triplicate at the recommended optimum operating settings. The experimental set up and procedures have been previously described (Section 4.4.3.3). Figure 4.4.6 illustrates the growth profiles of the hybridoma 192 cell in the 2 L bioreactor at the recommended optimum point. The growth profile is fairly typical with a sharp decline in the viable cell density after the maximum density is achieved. The extended stationary phase seen in some cell cultures does not occur.

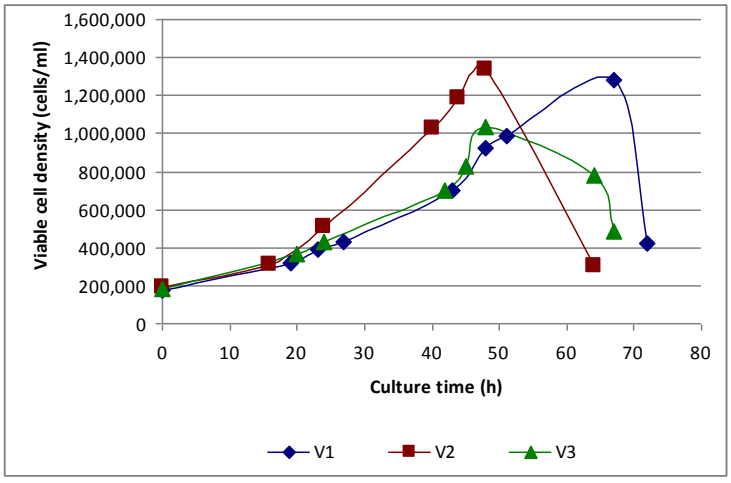


Figure 4.4.4: Growth profiles of hybridoma 192 cell in the 2L bioreactor at the recommended optimum point (0.09 vvm aeration rate, 100 rpm agitation speed, pH of 7.4, 36.8°C, and 30% dissolved oxygen level)

The MAb production profiles for the optimal culture conditions (Figure 4.4.4) are shown in Figure 4.4.5. The profiles are typical for this cell line growing in the bioreactor. The MAb titer increased slowly during the lag and exponential growth phases and once the cells entered the death phase, the MAb titer increased sharply. Table 4.4.8 compares the predicted and the actual values of specific growth rate, the maximum viable cell density, the maximum MAb titer and the average MAb production rate. Even though the predictive power of the models was expected to be poor based on the Q^2 values, they actually predicted the optimum responses reasonably well (Table 4.4.8). Only the standard deviation values of the maximum MAb titer for the three runs and the average MAb production rate were relatively high. The factor with an actual value that deviated the most from the predicted value was the maximum viable cell density (Table 4.4.8). In summary, the models obtained using Design Expert could predict rather well the experimental response.

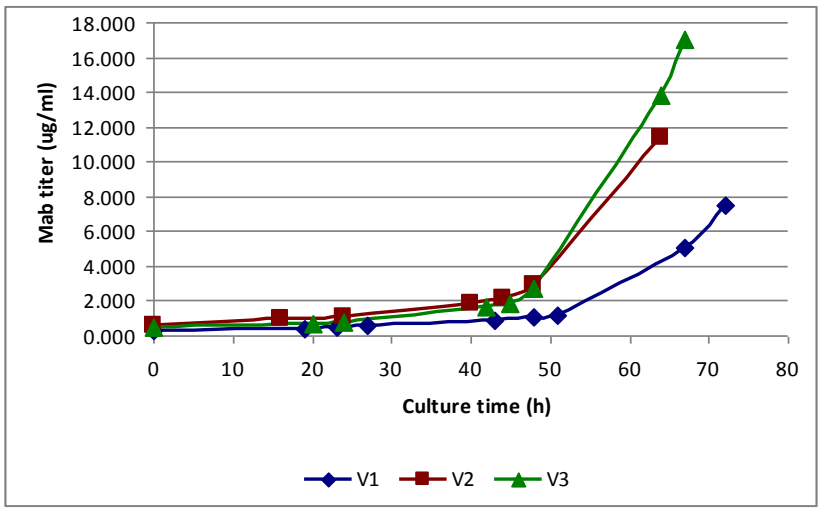


Figure 4.4.5: MAb production profiles of hybridoma 192 cell in the 2L bioreactor at the recommended optimum point

Table 4.4.8: Comparison between predicted and actual values

Factors	Predicted value	Actual value	% Deviation
Specific growth rate (1/h)	0.0368	0.0363 ± 0.0041	1.3
Maximum viable cell density (cells/mL)	1.93×10^6	$1.34 \pm 0.21 \times 10^6$	44.4
Maximum MAb titer (µg/mL)	12.000	11.941 ± 4.814	0.5
Average MAb production rate (pg/cell.h)	0.2166	0.2730 ± 0.1348	20.7

Standard deviation in a biological experiment is normally higher than in experiments involving purely physical phenomena. This is owing to the difficulty in producing exactly identical starting cells in different runs. The high standard deviation of the maximum MAb titer, the average specific MAb production rate and the maximum viable cell density in the above experiments may be attributable to the following reasons. Firstly, the maximum viable cell density that can be achieved in a cell cycle is highly dependent on the inoculum's age, the initial cell density and the initial total nutrients available in the culture medium. The inoculum age varies from batch to batch and cannot be predicted accurately. Similarly, the initial cell density of the culture in a bioreactor is hard to control at an exact value. This is because in order to get a fixed final total volume in the bioreactor, the initial culture volume in the bioreactor and the volume of inoculum have to be fixed. In addition, the viable cell density in the inoculum is slightly different in different runs at the time of inoculation because of variations in inoculum's age. Thus, the initial cell density does vary and the initial nutrient concentration in the culture medium also varies somewhat. As a result, the maximum viable cell density that can be achieved in a given run varies with a rather large standard deviation.

Variations in the maximum viable cell density that directly affected the maximum MAb titer attained at the end of the culture. Therefore, the maximum MAb titer obtained for each run was different. Furthermore, ELISA analysis itself had a rather high coefficient of variation (CV) in the intra- and inter-assays. Although the reported results are the average of duplicate or more plates, these high CV also contributed to the high standard deviation. As the average specific MAb production rate

was calculated based on the data of MAb titer, it too was influenced by the accuracy of the MAb titer measurements.

The optimum growth temperature for various types of hybridoma cells has been commonly reported to range from 31 to 34°C in serum-containing static and stirred cultures (Bloemkolk *et al.*, 1992; Reuveny *et al.*, 1986b; Sureshkumar and Mutharasan, 1991). In contrast, the present study showed that the optimum temperature for cell growth of hybridoma 192 was at 36.8°C. This was also the optimum temperature for the MAb production in agreement with the findings of Barnabé and Butler (1994), Bloemkolk *et al.* (1992) and Reuveny *et al.* (1986b).

The optimum pH for both cell growth and MAb production was at pH 7.4. A similar observation was reported by Miller *et al.* (1988a) for continuous culture of hybridoma AB2-143.2 in serum-containing DMEM. In contrast, Ozturk and Palsson (1991a) reported an optimum pH of 7.2 for the hybridoma 167.4G5.3 cultured in a 1.5 L batch bioreactor.

The optimum stirring speed of 100 rpm (impeller tip speed = 0.288 ms^{-1}) obtained in this study, was consistent with the optimum value reported by Griffiths (2000) for suspension cells. Similarly, the optimum sparging rate of 0.09 vvm used in this study was within the range used by Michaels *et al.* (1996). Michaels *et al.* (1996) reported that cells were able to grow at the normal rate at a sparging rate as high as 0.15 vvm.

The optimum parameter settings obtained in this study were in agreement with Yang *et al.* (2007), who scaled up production of Epratuzumab antibody from 3 L to 2,500 L. The full scale process was operated at 37°C, 40% DO and pH 7.3 using Sp2/0 mouse myeloma cells. Similar operational settings were used by Chen *et al.* (2000) in a 5 L bubble free aeration bioreactor (agitating at 22 rpm, 37°C, 40% DO, pH 7 – 7.2) to produce human prothrombin using CHO cells. Gaertner and Dhurjati (1993) reported that a high rate of hybridoma cell growth and antibody production could be attained at 37°C, between pH value of 7.3 and 7.4, and with a low DO concentration. It appears that the operating parameters in bioreactors for most animal cell types are rather similar.

4.4.4.5 Comparison of the cell growth and MAb production in T-flasks, spinner flasks and the bioreactor

Table 4.4.9 compares cell growth and MAb production in T-flasks, spinner flasks and the bioreactor. Specific growth rates in spinner culture and the bioreactor were comparable, but slightly higher than in the T-flask culture. The maximum viable cell density in the spinner culture was the highest of the three culture systems and was lowest in the bioreactor. Even so, the maximum viable cell density that could be achieved in the bioreactor was quite comparable to that in the static T-flask. Thus, the use of stirring and sparging did not significantly affect the growth of the hybridoma 192 in comparison with the static culture.

Table 4.4.9: Comparison of the responses for T-flasks, spinner flasks (100 mL, 40 rpm) and the bioreactor

Factors	T-flask[1]	Spinner flask[1]	Bioreactor[2]
Specific growth rate (1/h)	0.0330 ± 0.0014	0.0380 ± 0.0020	0.0363 ± 0.0041
Maximum viable cell density (cells/mL)	$1.48 \pm 0.07 \times 10^6$	$1.77 \pm 0.01 \times 10^6$	$1.34 \pm 0.21 \times 10^6$
Maximum MAb titer (µg/mL)	4.436 ± 3.767	5.363 ± 1.141	11.941 ± 4.814
Average MAb production rate (pg/cell.h)	0.0569 ± 0.0453	0.1436 ± 0.0451	0.2730 ± 0.1348

[1] Average of quadruplicate runs
[2] Average of triplicate runs

There was a great improvement of maximum MAb titer and the average MAb production rate in moving from the T-flask culture to the optimized bioreactor culture (Table 4.4.9). Although the maximum MAb titer in the T-flask culture was comparable to the spinner culture, the standard deviation was large. In the bioreactor, the maximum MAb titer attained was about 3-fold greater than in the T-flask culture. The optimized conditions in the bioreactor also enhanced the average MAb production rate by 4.8-fold as compared to the T-flask culture. Even though the specific growth rate in the

bioreactor was similar to the values in the other culture systems, the production of MAb was greatly enhanced. A similar observation was reported by Miller *et al.* (1988a) and Luo and Yang (2004).

4.4.5 Conclusion

The operating parameters of the 2 L bioreactor were successfully optimized within the range tested using statistical experimental design. The optimum settings for the 2 L bioreactor were determined to be 36.8°C, pH 7.4, stirring speed of 100 rpm, 30% dissolved oxygen and 0.09 vvm aeration rate. The maximum viable cell density achieved was $1.34 \pm 0.21 \times 10^6$ cells/mL with a specific growth rate of 0.0363 ± 0.0041/h. The maximum MAb titer obtained was 11.941 ± 4.814 μg/mL with the average specific MAb production rate of 0.2730 ± 0.1348 pg/cell.h. The growth of hybridoma 192 in bioreactor was comparable to that in the T-flask culture, but there was a 3-fold increment in the MAb titer. In the bioreactor, the average specific MAb production rate was 4.8-fold greater than in the T-flask.

4.5 Scale Up To 20 L Pilot Scale Bioreactor

4.5.1 Introduction

Generally, the animal cell culture bioreactors are scaled up in stages such that the volume ratio between one stage and the next is 1:10. This approach was used here to scale up from the 2 L scale to 20 L. The scale up strategy was based on maintaining an equal impeller tip speed at the two scales (Garchia-Ochoa and Gomez, 2009). A 20 L pilot scale bioreactor was used without any change to the original design. The only change was the height of the impeller from the bottom of the vessel. This height was adjusted to 10.5 cm to meet the geometrical similarity requirement with respect to the 2 L bioreactor. Thus, D_T/H_i ratio was kept at 2.53 at both scales. In other respects, the two bioreactors did not conform to an exact geometry similarity.

4.5.2 Objectives of study

The objective of the study was to examine the ability of the hybridoma 192 cells to grow and produce monoclonal antibody at the 20 L scale in a way that was comparable to that at the 2 L scale.

4.5.3 Materials and methods

4.5.3.1 Geometry of 2 L and 20 L bioreactors

The details of geometry of the 2 L bioreactor and the 20 L bioreactor are provided in Table 4.5.1. The bioreactors and the experimental set up are shown in Appendix B, Figure B7.

Table 4.5.1: Details of geometry of 2 L and 20 L bioreactors

Size of bioreactor	2 L	20 L
Impeller type	Marine	Pitched Paddle
Impeller diameter, D_i (cm)	5.5	15.0
Vessel type	Double jacketed glass	Double jacketed SS 316
Vessel inner diameter, D_T (cm)	11.9	26.6
Sparger type	Single hole	Ring (10 holes)
Sparger hole diameter (mm)	3	1
Impeller height from the vessel bottom, H_i (cm)	4.7	10.5
Medium height from the vessel bottom, H_m (cm)	11.3	25.3

4.5.3.2 Scale up strategy

Owing to the shear sensitivity of hybridoma 192 cells, the scale up strategy used was based on keeping an equal impeller tip speed at the two scales. The two bioreactors were not exactly geometrically similar. Nevertheless, a close geometric similarity was attained for practical purposes (Table 4.5.2). Thus, the ratio of medium depth (H_m) to the vessel diameter (D_T) was identical (Table 4.5.2). The total volume needed at 20 L scale was then estimated based on the dimensions obtained. The ratio of impeller clearance from the bottom (H_i) to the culture depth (H_m) was also identical. In addition, the D_i/D_T ratios were fairly close at the two scales. The optimum temperature (36.8°C), pH (7.4), dissolved oxygen (30%) and volumetric gas flow rate (0.09 vvm) were kept identical at the two scales. Stirring speed in 20 L pilot scale bioreactor was calculated to be 37 rpm. This gave the same impeller tip speed ($\pi N D_i$) at the two scales. (The impeller rotational speed at 2 L scale was 100 rpm.)

Table 4.5.2: Geometrical similarities between 2 L and 20 L bioreactors

Size of bioreactor	2 L	20 L
D_i / D_T	0.462	0.565
H_m / D_T	0.95	0.95[a]
H_i / H_m	0.416	0.416[b]

[a] Used to calculate the volume of the medium required
[b] Used to calculate the height of the impeller from bottom of the vessel

4.5.3.3 Cell culture procedures

The pilot scale 20 L bioreactor used in this study was Biostat C (Sartorius Stedim, Germany), which had an in-situ sterilization capability. Cells were thawed in T-flasks and subcultured into spinner flasks. The cells were then inoculated into the 2 L bioreactor (Biotron, Korea) run at 1.25 L working volume of the culture medium. The operational parameter settings were as determined in Section 4.4 (i.e. 36.8°C, pH 7.4, 30% dissolved oxygen level, 0.09 vvm aeration rate). A 25 mL sample was withdrawn at 0, 24 and 50 h to monitor the cell growth. 0.90 L of fresh medium was fed to the bioreactor right after sampling at 50 h using a peristaltic pump (Watson Marlow). At 72 h, a cell count was done. If the viable cell density was within $1.2 – 1.4 \times 10^6$ cells/mL, the broth was inoculated into the 20 L bioreactor using a peristaltic pump. The 20 L pilot scale bioreactor was run at 14 L working volume of culture medium at 36.8°C. The oxygen level was maintained at 30% of air saturation by sparging either pure oxygen or pure nitrogen into the culture through a ring sparger at a rate of 1.26 L/min (0.09 vvm), as needed. The pH was controlled at 7.4 by the use of CO_2 and 7.5% $NaHCO_3$. Stirring speed was maintained at 37 rpm using a single pitched paddle impeller (Figure C8). 70 mL samples were taken 2 - 3 times a day until cell viability dropped to 30% or below. On harvesting, 4 L samples were taken for purification of the MAb as described in Chapter 3 in order to determine the MAb specificity and confirm its structural integrity.

4.5.4 Results and discussion

4.5.4.1 Growth and production profiles

Figure 4.5.1 and Figure 4.5.2 show the growth curves and the MAb production profiles of cells in the 20 L bioreactor for 3 independent runs, respectively. The growth and production patterns were exactly the same as were observed in the 2 L bioreactor. For example, the cells entered the death phase right after achieving the maximum viable cell density and the MAb titer increased rapidly in the death phase.

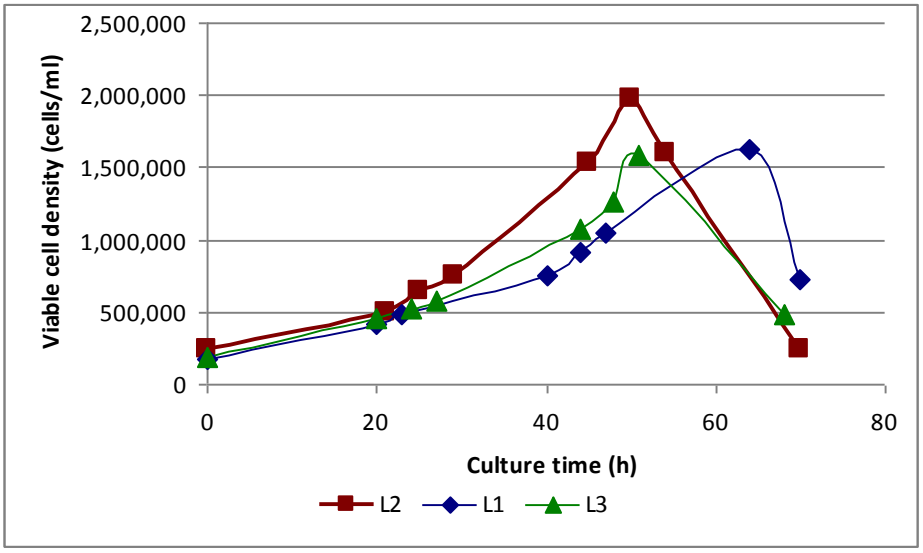


Figure 4.5.1: Growth profiles of cells (3 independent runs) in the 20 L bioreactor

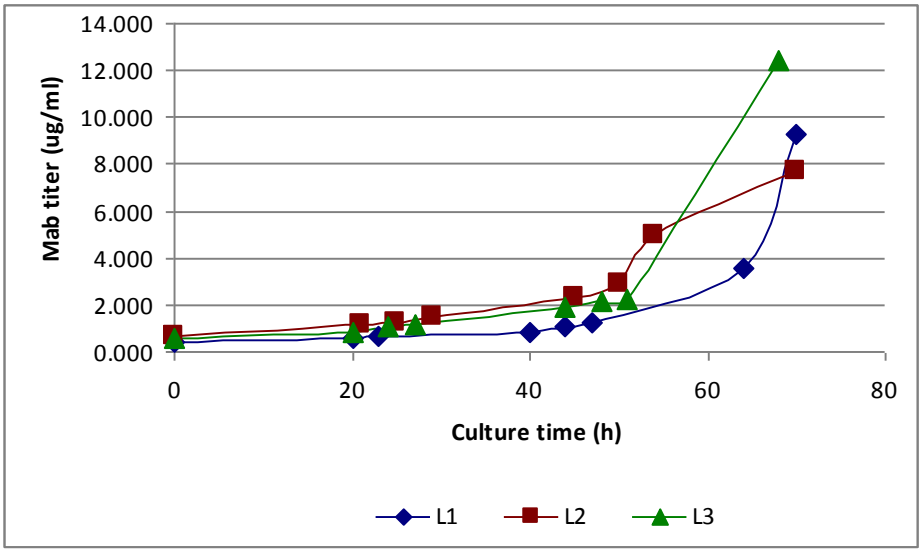


Figure 4.5.2: MAb production profiles (3 independent runs) in the 20 L bioreactor

Table 4.5.3 compares the performance of the 2 L and 20 L bioreactors in terms of the average values of the maximum viable cell density attained, specific growth rate, maximum Mab titer achieved and the average specific MAb production rate. Surprisingly, the maximum viable cell density achieved at 20 L scale was about 40% higher than that achieved at the 2 L scale. The specific growth rate of the hybridoma was also slightly higher in the 20 L bioreactor. However, owing to the negatively growth-associated MAb production characteristic of the hybridoma 192, the maximum MAb titer attained and the average specific MAb production rate were lower at the larger scale. The maximum MAb titer achieved at 20 L scale was about 82% of the value attained at the smaller scale. Similarly, the average specific MAb production rate at the 20 L scale was 62% of the value attained at the 2 L scale.

Table 4.5.3: Comparison of results between the 2 L bench scale and the 20 L bioreactors

Scale of bioreactor	**2 L**	**20 L**
Maximum viable cell density (cells/mL)	$1.340 \pm 0.210 \times 10^6$	$1.890 \pm 0.125 \times 10^6$
Specific growth rate (1/h)	0.0363 ± 0.0041	0.0401 ± 0.0012
Maximum MAb titer (µg/mL)	11.941 ± 4.814	9.782 ± 2.417
Average specific MAb production rate (pg/cell.h)	0.2730 ± 0.1348	0.1695 ± 0.0599
Specific glucose consumption rate (mmol/10^9 cell.h)	0.465 ± 0.037	0.399 ± 0.068
Specific lactate production rate (mmol/10^9 cell.h)	0.766 ± 0.095	0.664 ± 0.091
Specific glutamine consumption rate (mmol/10^9 cell.h)	0.117 ± 0.014	0.089 ± 0.015
Specific ammonia production rate (mmol/10^9 cell.h)	0.087 ± 0.030	0.041 ± 0.006
Yield of lactate on glucose	1.65	1.66
Yield of ammonium on glutamine	0.74	0.46

As previously mentioned, the 2 L and 20 L bioreactors used in this study were not exactly geometrically similar (Table 4.5.1). Both were commercial stirred tank bioreactors. They had somewhat different impellers and gas spargers. The 2 L bioreactor used a marine impeller and a single hole sparger, whereas the 20 L bioreactor used a pitched paddle impeller and a multi-hole ring sparger. The sparger in the 2 L bioreactor generated bubbles that were ~ 3 mm in diameter. In contrast, the sparger in the 20 L bioreactor generated bubbles of 1 mm size. The impellers in both bioreactors were of the axial flow type, which guarantee less shear stress and are suitable for plant and mammalian cell cultures (Jain and Kumar, 2008; Griffiths, 2000). Although the bioreactors in this study generated gas bubble of 1 – 3 mm diameter, which were within a size range that is considered to be damaging to cells (Chisti, 2000), no significant damaging effect was seen. In fact, the cell growth was improved at the 20 L scale. Therefore, a reduced bubble size at the 20 L scale did not seem harmful to hybridoma 192.

Gas-liquid mass transfer coefficient (k_La) was measured in the two bioreactors in cell-free culture medium using the dynamic method (Garchia-Ochoa and Gomez, 2009). The results are shown in Table 4.5.4. The k_La value for the 20 L bioreactor was doubled that of the value for 2 L bioreactor. Therefore, with the same level of 30% dissolved oxygen in the two bioreactors, the rate of oxygen transfer in the 20 L bioreactor was twice as high as in the 2 L bioreactor. In the larger bioreactor, the cells would not become oxygen limited even if the aeration rate was reduced a little, for example. This may have been a possible reason for the improved specific growth rate and the maximum viable cell density at the 20 L scale. According to Garchia-Ochoa and Gomez (2009), scale up based on equal impeller tip speed results in a lower k_La value at the larger scale. This was not the case in this study. However, this may be due to the different types of impellers and spargers used at the two scales.

Table 4.5.4: Comparison of k_La values in the two bioreactors

Scale of bioreactor	**2 L***	**20 L[#]**
k_La (1/s)	0.0006 ± 0.0000	0.0012 ± 0.0000

* Average of triplicate runs

[#] Average of duplicate runs

Table 4.5.3 compares the metabolites consumption / production rates of the 2 L and the 20 L bioreactors. The specific glucose consumption and specific lactate production rates for 20 L bioreactor were slightly lower, but generally comparable to those of the 2 L bioreactor. A possible improved availability of oxygen to the cells as discussed above may have reduced the glycolysis flux, which reduced the specific glucose consumption rate and specific lactate production rate. (This is the well-known Pasteur Effect (McKee and McKee, 2003).) The specific glutamine consumption rate and the specific ammonia production rate, on the contrary, were much lower at the 20 L scale compared to the 2 L scale. According to Oh *et al.* (1992), the metabolic rate of cells is higher under stress conditions. Since the rate of metabolism was reduced, it appears that the stress experienced by cells is less in the 20 L bioreactor than that in 2 L bioreactor. As more energy was diverted for growth than for MAb production, the specific glutamine consumption rate was reduced. Following this, the specific ammonia production rate was also reduced. No changes were observed in the yield of lactate from glucose when the culture was scaled up to the 20 L bioreactor. However, the yield of ammonium from glutamine decreased. It was likely that more glutamine was used to produce cells in the 20 L bioreactor compared to the case in the 2 L bioreactor (Hayter *et al.*, 1992).

4.5.4.2 Specificity of the MAb produced

As described in Section 3.4.2, the culture broth samples were purified using the Protein G column chromatography. The material eluted from the column was collected in 1 mL fractions. The optical density (OD) of each fraction was measured at 280 nm using UV-Vis spectrophotometer to identify which fractions contained the antibody. Figure 4.5.3 shows the optical density versus fraction number chromatogram. Fraction 3, 4 and 5 that had the most antibody protein were pooled and desalted. The resulting sample was then used for the specificity test and SDS-PAGE.

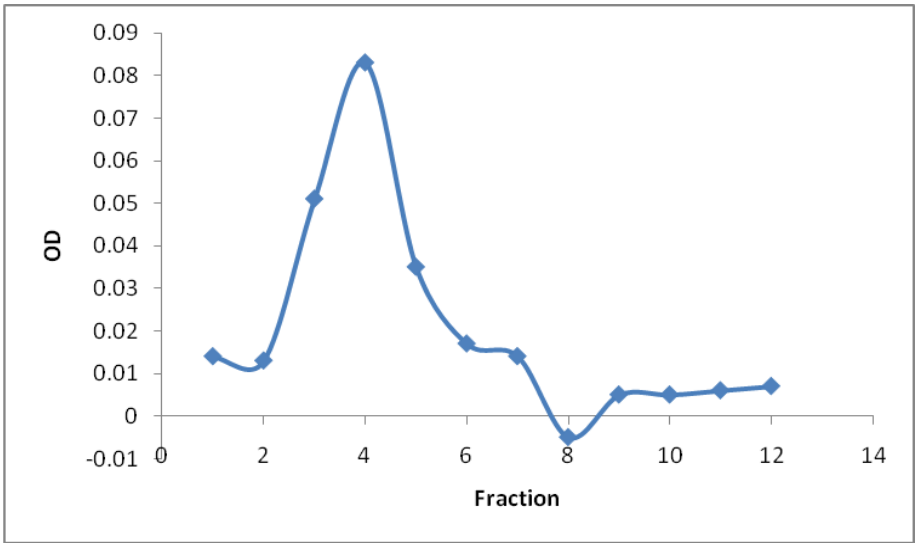


Figure 4.5.3: Chromatogram of purified sample at 280 nm

Figure 4.5.4 illustrates the plot of OD versus dilution factor for the MAb sample. This plot was used to determine the best dilution factor for use in specificity test. Dilution factor which gave 80% of the saturation OD was taken to be the best as is typical for such assays. From Figure 4.5.4, the best dilution factor was identified to be 0.0625 or 16×.

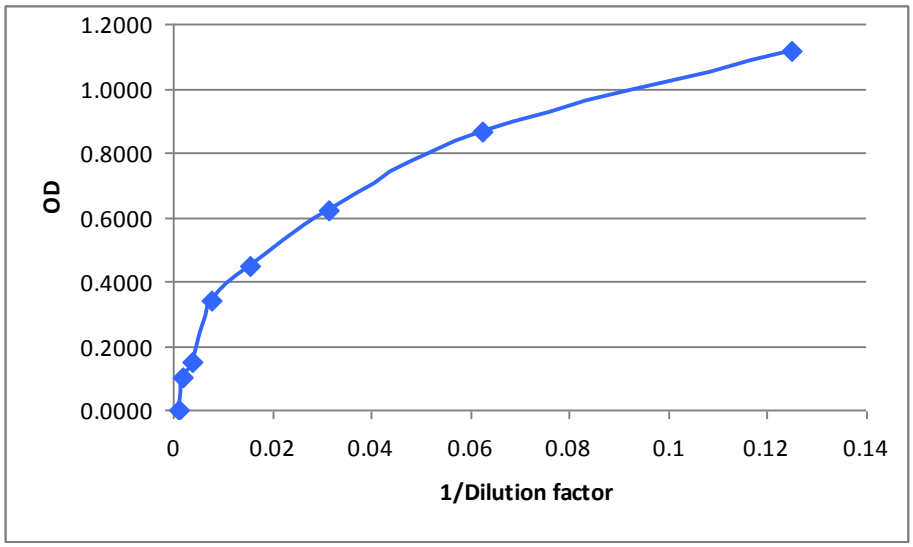


Figure 4.5.4: Plot of OD versus 1/dilution factor for the purified MAb from the 20 L bioreactor

This concentration of purified MAb was used to run the specificity test as described in Section 3.6.2. Figure 4.5.5 shows the results of the specificity test using free 17-OHP. For a fixed MAb concentration in the sample, the optical density decreases with increasing concentration of the free antigen (17-OHP) as the antibody binds to this antigen.

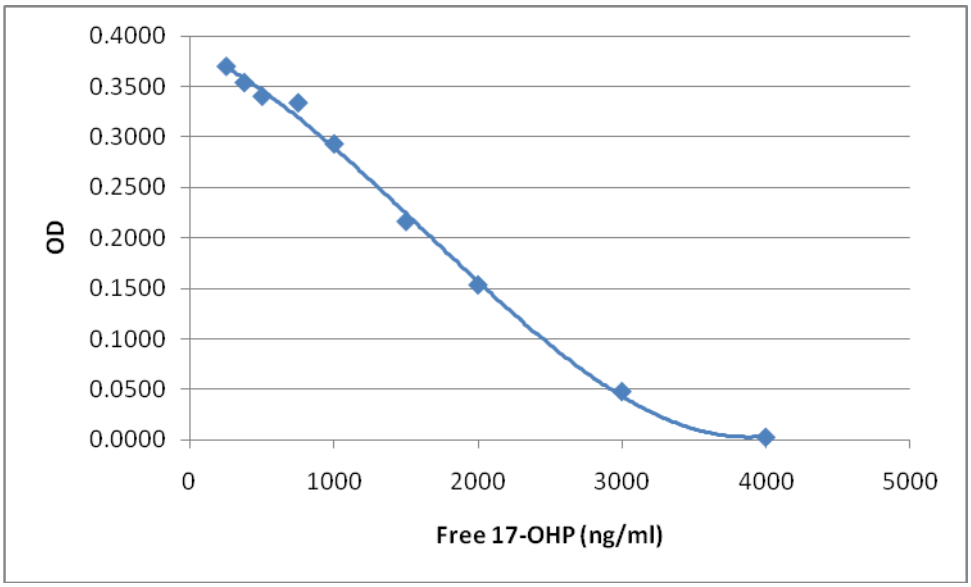


Figure 4.5.5: Specificity test with purified sample from 20 L bioreactor

4.5.4.3 Structural integrity of the MAb produced

It is important to check that in moving from the serum-containing medium to LSD, and from static culture to the stirred culture of the 20 L bioreactor, the change of culture environment and medium have not affected the structural integrity of the MAb produced by the hybridoma 192. A MAb with an altered structure may not be effective in diagnosing CAH, or detecting 17-OHP, in clinical use. Therefore, SDS-PAGE was used to check for the MAb's structural identity and purity (Yang *et al.*, 2007; Schenerman *et al.*, 1999).

The MAb produced by hybridoma 192 that recognizing 17-OHP is a type of IgG (immunoglobulin G). IgG differs from other immunoglobulin (i.e. IgA, IgE, IgD and IgM) by their type of heavy chains. The heavy chain in IgG is of the γ type. Since hybridoma 192 resulted from the fusion of mouse myeloma cells (P3X63 Ag8.653) and

mouse lymphocytes, the structure and molecular weight of the MAb is expected to closely resemble that for the mouse IgG. Therefore, standard mouse IgG was used as a reference in this study.

Standard mouse IgG was run through a non-reducing and reducing gel for use as a guide and for comparison with the MAb sample obtained from the 20 L bioreactor. Figure 4.5.6 shows the gel that was stained with Coomasie stain. Non-reducing sample in Figure 4.5.6 shows that standard mouse IgG had a molecular weight of about 150 kDa. This IgG was reduced to two chains, the heavy chain and the light chain, on exposure to the reducing agent (i.e. 2-mercaptoethanol). Heavy chain had a molecular weight of around 50 kDa and the light chain was about 22 kDa in molecular weight.

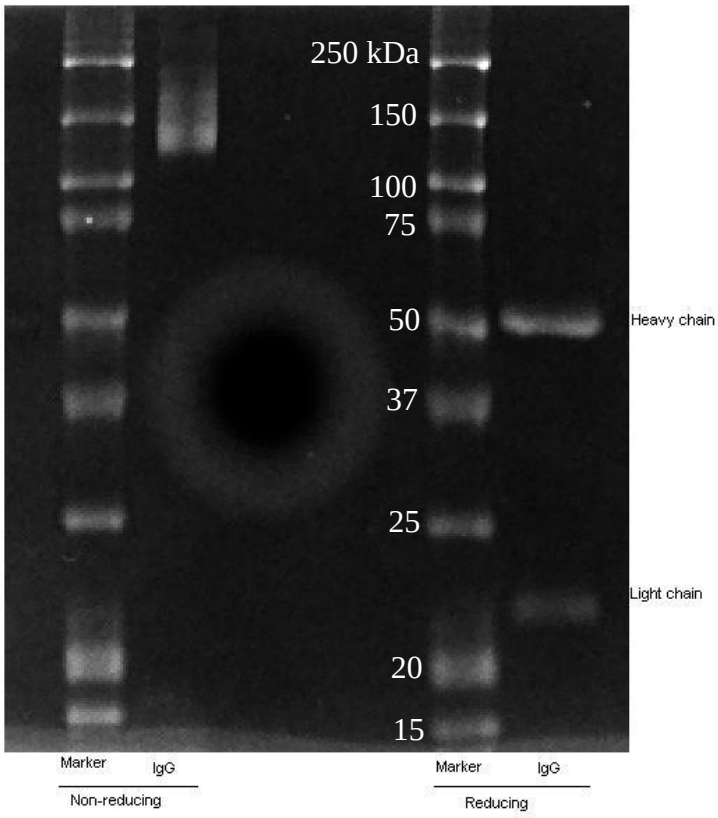


Figure 4.5.6: Coomasie stained gel with reducing and non-reducing mouse IgG standard

Owing to the low concentration of the MAb obtained in the 20 L bioreactor's sample even after purification, silver staining was used to stain the gel instead of Coomasie staining. Silver staining is known to be more sensitive than Coomasie staining and allows determination of protein at concentrations in the nanogram range

(Morthz *et al.*, 2001). Figure 4.5.7 shows the silver stained gel loaded with the markers (Precision Plus Protein Kaleidoscope standards, BioRad), the standard mouse IgG and the partially purified MAb sample from the 20 L bioreactor. In Figure 4.5.7, the standard mouse IgG used was seen to be not 99.9% pure as there were some other bands beside those at 50 and 22 kDa. However, these two bands were clearly the dominant ones. Because of the sensitivity of silver staining, the impurity in the standard mouse IgG could be seen in this gel. (The standard mouse IgG used in this study was only 95% pure (Sigma's product specification sheet).) The MAb or IgG produced by the hybridoma 192, which is a mouse-mouse hybridoma cell line, resembled the structure of the mouse IgG. Therefore, the MAb obtained from the 20 L bioreactor showed two heavy and light chains at the same molecular weight as those for the standard mouse IgG. There were also some other bands, which obviously were the impurities in the sample, as only a partially purified sample was used. Therefore, the structure of the MAb appeared to be consistent with expectation. The MAb produced in the 20 L bioreactor was able to recognize its antigen well (Section 4.5.4.2).

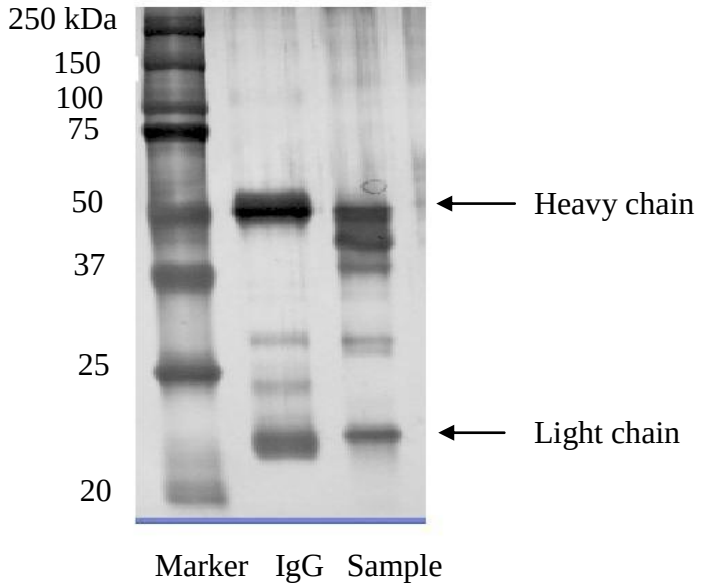


Figure 4.5.7: Silver stained gel with the reduced MAb sample from the 20 L bioreactor and the reduced mouse IgG standard

4.5.5 Conclusion

Scale up of hybridoma 192 culture from the 2 L bench scale bioreactor to the 20 L bioreactor was successfully achieved using the constant impeller tip speed as the scale up criterion. A specific growth rate of 0.401/h and a maximum viable cell density of 1.89×10^6 cells/mL were achieved in the 20 L bioreactor. These values were higher than those in the 2 L bioreactor. However, the maximum MAb titer in the 20 L bioreactor was 18% lower than in the 2 L bioreactor. After going through several modifications in the cells' environment from static to stirred culture, the MAb produced by hybridoma 192 is still specific to its antigen, 17-OHP. In addition, the scale up process did not compromise the structural integrity of the MAb.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Introduction

The main objective of this study was to develop and scale-up a submerged culture process for the production of a diagnostic monoclonal antibody (MAb) against Congenital Adrenal Hyperplasia (CAH) using the hybridoma 192 cell line. The intention was to establish a process that could provide a substantial quantity of the MAb inexpensively. Studies included measurement of the baseline data in static culture and the bioreactor; optimization of a low serum medium with inexpensive supplements; identification of possible inducer agents; operational parameters optimization in the bench scale bioreactor using a design of experiment approach; and scale up to 20 L bioreactor. A summary of the outcomes of the study and its main conclusions are provided in the following sections.

5.2 General Conclusions

5.2.1 Serum Reduction and Baseline Data Collection

Hybridoma 192 could be grown in a low-cost low-serum medium. The specific growth rate and the average specific MAb production rate in the new medium were comparable to data obtained in DMEM supplemented with 5% FBS, 1% antibiotic-antimycotic and 4 mM L-glutamine. Fed-batch cultivation of cells in spinner flasks demonstrated that an improved oxygen supply could improve the specific growth rate relative to static cultures. In addition, use of the fed-batch operation enhanced the total maximum viable cells numbers attained and extended the culture lifespan. However, this was at the expense of the average specific MAb production rate. The growth rate of the hybridoma 192 was reduced when cultivated in a sparged and stirred bioreactor, possibly because of the shear effects, but the average specific MAb production rate was improved. This was because of an inverse relationship between the growth rate and the MAb production rate.

5.2.2 Medium Optimization Using Statistical Experimental Design

By using statistical experimental design, the serum content in the culture medium for the hybridoma 192 was successfully reduced to 0.4% and the medium was optimized with inexpensive supplements (ferric citrate, zinc sulfate, sodium selenite). This new formulation cut down the cost of the medium by nearly 70% compared to the 2% DMEM medium and nearly 87% compared to the original medium (DMEM + 5% FBS) cost. The average specific MAb production rate and the maximum MAb titer were greatly enhanced in the new LSD medium. Furthermore, the activity of the MAb produced was not affected by the changed medium.

5.2.3 Screening and Optimization of Inducer Agents

Of the potential inducer agents screened, only LPS induced the production of the MAb. Optimization through statistical experimental design identified an LPS concentration of 8 μg/mL to be the optimum for enhancing the MAb production. A confirmation run verified that LPS increased the average specific MAb production rate by 1.6-fold compared to the control culture. However, no improvement in the maximum MAb titer was achieved. The results were in contrast to those obtained during the screening and optimization processes. Therefore, the performance of LPS as an inducer agent was inconsistent. Since LPS could not enhance MAb production by greater than 2-fold, but increased the medium cost by as much as 40-fold, its use was not cost-effective and was not further pursued.

5.2.4 Operational Parameter Optimization Using Statistical Experimental Design

The optimum operating parameters for the 2 L bioreactor were determined using statistical experimental design. Under the optimal conditions (36.8°C, pH 7.4, stirring speed of 100 rpm, 30% dissolved oxygen, aeration rate of 0.09 vvm), the maximum viable cell density achieved was 1.34×10^6 cells/mL, which was a little lower than in the T-flask culture. The specific growth rate of hybridoma 192 in the bioreactor was slightly improved compared to the T-flask culture due to improved oxygen transfer and

better mixing. The greatest enhancement obtained through optimization process was the 4.8-fold increase of the average specific MAb production rate in the bioreactor compared to the T-flask culture. The maximum MAb titer in the bioreactor increased by about 3-fold compared to the T-flask culture. The stress associated with gas sparging and stirring was believed to contribute to this improved MAb production.

5.2.5 Scale Up to 20 L Pilot Scale Bioreactor

Production of the MAb by the hybridoma 192 was successfully scaled-up to a 20 L bioreactor using a constant impeller tip speed as the scale up criterion. The hybridoma 192 was found to be shear sensitive. The 20 L bioreactor achieved better oxygen transfer compared to the 2 L bioreactor. As a result, the specific growth rate and the maximum viable cell density were higher in the 20 L bioreactor compared to the 2 L bioreactor. The maximum MAb titer in the larger bioreactor was 82% of the value obtained in the 2 L bioreactor due to the negatively-growth associated production characteristics. The MAb produced in the 20 L bioreactor recognized well its antigen, 17-OHP. In addition, the structure of the MAb was unaffected by the changes in the cells' environment due to scale up and medium optimization.

5.2.6 Overall achievement

Overall, the culture of hybridoma 192 was successfully optimized and scaled-up to a 20 L bioreactor without impairment of the product quality. A total medium cost saving of 87% was achieved. The MAb titer obtained at harvest was greatly improved (12-fold enhancement) compared to T-flask culture with DMEM + 5% FBS. Similarly, in the bioreactor, there was a 29-fold enhancement of the average specific MAb production rate compared to T-flask culture.

5.3 Recommendation for further work

An initial objective of this study was to totally wean off the cells from a dependency on serum in order to reduce the medium cost and/or production cost. Although the LSD medium developed in this study supported the growth of hybridoma 192, it did require some serum and this serum content contributed 60% of the medium cost. Furthermore, the average specific MAb production rate was still far lower than the literature values reported for other hybridoma cell lines. Since hybridoma 192 produced more MAb under stress conditions and MAb production was negatively-growth associated, a method should be found to grow the cells at a slower rate to a high number with improved viability and then suppress growth by introducing some stress factor and induce production of the MAb. Therefore, the following studies are recommended to further optimize the MAb production and to gain a better insight in the behavior of the hybridoma 192:

1. Investigation of the cell's requirements for glucose and glutamine for growth and MAb production. An optimization of the glucose and glutamine concentrations may further reduce the medium cost. In addition, the information obtained may prove useful in a possible fed-batch cultivation operation at the large scale.
2. Attempts should be made to totally wean the cells off serum by using a proper weaning protocol and considering other simple supplements such as non-essential amino acids and trace elements.
3. Investigate the relationship between the amino acids consumption and/or production and the rate of cell growth and MAb production. This information may be useful in a possible fed-batch culture.
4. A thorough investigation of the other possible stress factors (e.g. cheap and simple inducer agents, increase of medium osmolality) that could suppress growth and induce MAb production.
5. Other cultivation modes, such as fed-batch and perfusion culture may be worthwhile studying. These cultivation modes can prolong the culture lifespan and with suitably planned feeding have the potential to increase the MAb production.
6. Further studies are also required to more thoroughly assess product quality than was done in this work.

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APPENDIX A

RECIPES OF SOLUTIONS USED

Complete Media

Table A.1: Formula of complete media

Components	Type of media	
	2% DMEM	**LSD**
FBS	2% v/v	0.4% v/v
L-Glutamine	4 mM	4 mM
Antibiotic	1% v/v	1% v/v
Zinc sulfate	-	4.5 µM
Ferric citrate	-	311.8 µM
Sodium selenite	-	17.3 nM
Pluronic F-68	0.1% w/v	0.1% w/v

SDS-PAGE Gel Electrophoresis

Table A.2: Stock solutions required for electrophoresis

Solutions	Volume (mL)	Concentration	pH
Tris-HCl	100	2 M	8.8
Tris-HCl	100	1 M	6.8
Sodium dodecyl sulfate (SDS)	100	10% w/v	-
Glycerol	100	50% w/v	-
Bromophenol blue	10	1% w/v	-

Table A.3: Working solutions required for electrophoresis

Solutions	Formula	Volume (mL)
A	75 mL 40% (w/v) acrylamide 25 mL distilled water	100
B	75 mL 2 M Tris-HCl (pH 8.8) 4 mL 10% (w/v) SDS 21 mL distilled water	100
C	50 mL 1 M Tris-HCl (pH 6.8) 4 mL 10% (w/v) SDS 46 mL distilled water	100
10% (w/v) ammonium persulfate	0.5 g ammonium persulfate 5 mL distilled water	5
Electrophoresis buffer	6 g Tris 28.8 g glycine 2 g SDS in 2 L distilled water (pH ~8.3)	2000
Sample buffer	0.6 mL 1 M Tris-HCl (pH 6.8) 5 mL 50% glycerol 2 mL 10% SDS 0.5 mL 2-mercaptoethanol 1 mL 1% bromophenol blue 0.9 mL distilled water ***Note: Omit 2-mercaptoethanol when preparing non-reducing sample buffer***	10
Staining solution (Coomassie stain)	1 g Coomassie Blue R-250 450 mL methanol 450 mL distilled water 100 mL glacial acetic acid	1000
De-staining solution (Coomassie stain)	100 mL methanol 100 mL glacial acetic acid 800 mL distilled water	1000

Table A.4: Volume of various stock solutions required to prepare the separating and stacking gels

Solutions	10% Separating gel	5% Stacking gel
A	5 mL	0.67 mL
B	3.75 mL	-
C	-	1 mL
Distilled water	6.25 mL	2.3 mL
Ammonium persulfate	75 µL	30 µL
TEMED	25 µL	15 µL

Table A.5: Solutions required for silver staining

Solutions	Volume	Concentration
Ethanol : acetic acid: UP water	200 mL	40% : 10% : 50% (by volume)
Sodium thiosulfate	200 mL	0.02% w/v
Silver nitrate solution	200 mL	0.1% w/v (added with 0.02% v/v 37% formaldehyde)
Sodium carbonate	250 mL	3% w/v (added with 0.05% v/v 37% formaldehyde)
Acetic acid	200 mL	5% v/v

APPENDIX B

GEOMETRIC DETAILS AND PHOTOS OF SPINNER FLASK AND BIOREACTOR

Figure B1: 100 mL spinner flask with single pendulum

Figure B2: 250 mL spinner flask with dual pendulums

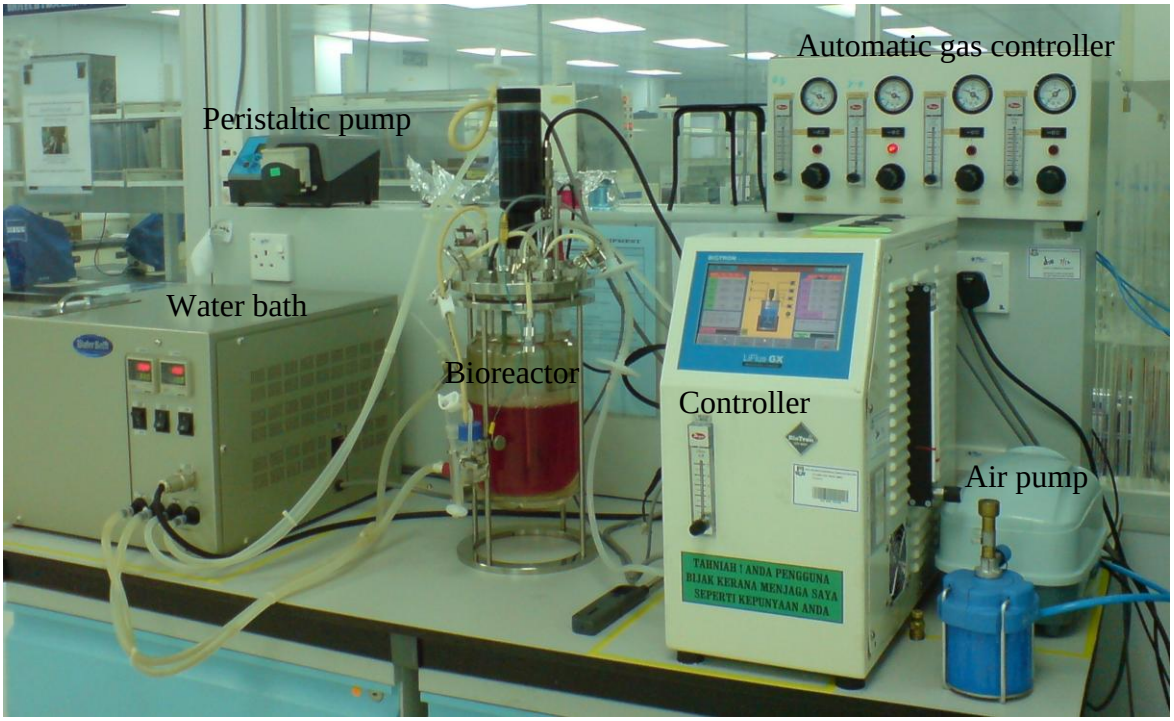

Figure B3: 2L scale bioreactor system

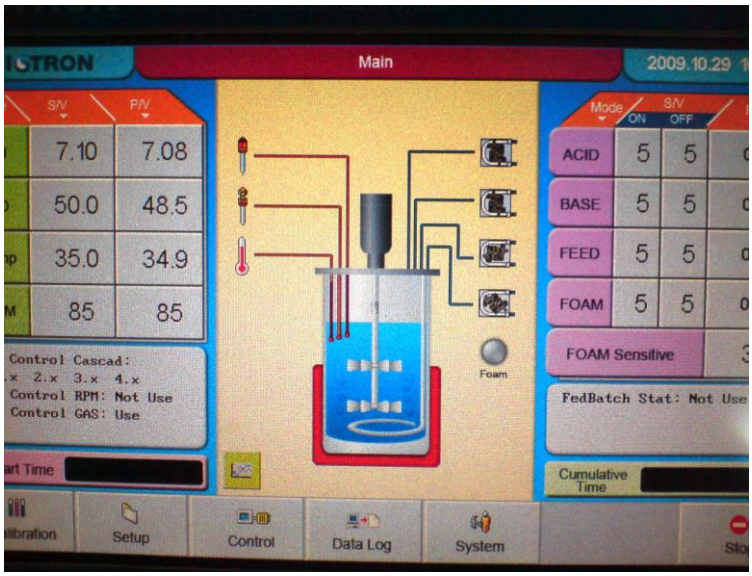

Figure B4: Interface of controller for 2L scale bioreactor

Figure B5: Data acquisition system for 2L bioreactor

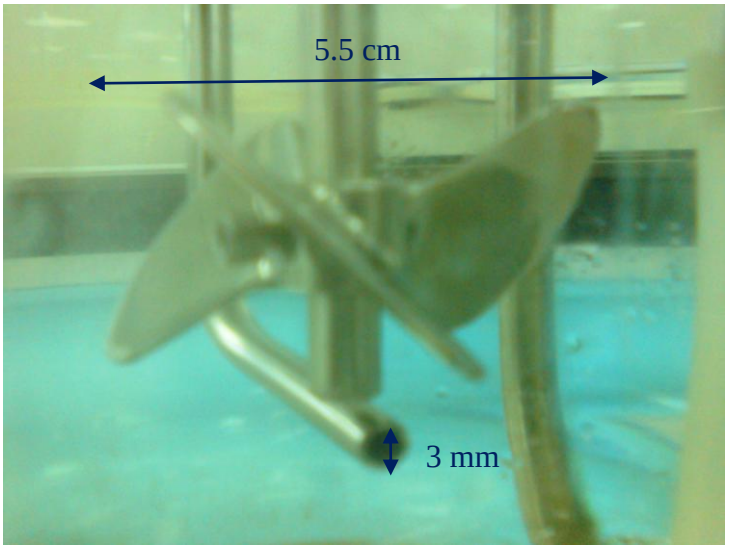

Figure B6: Type and dimension of impeller in 2L bioreactor

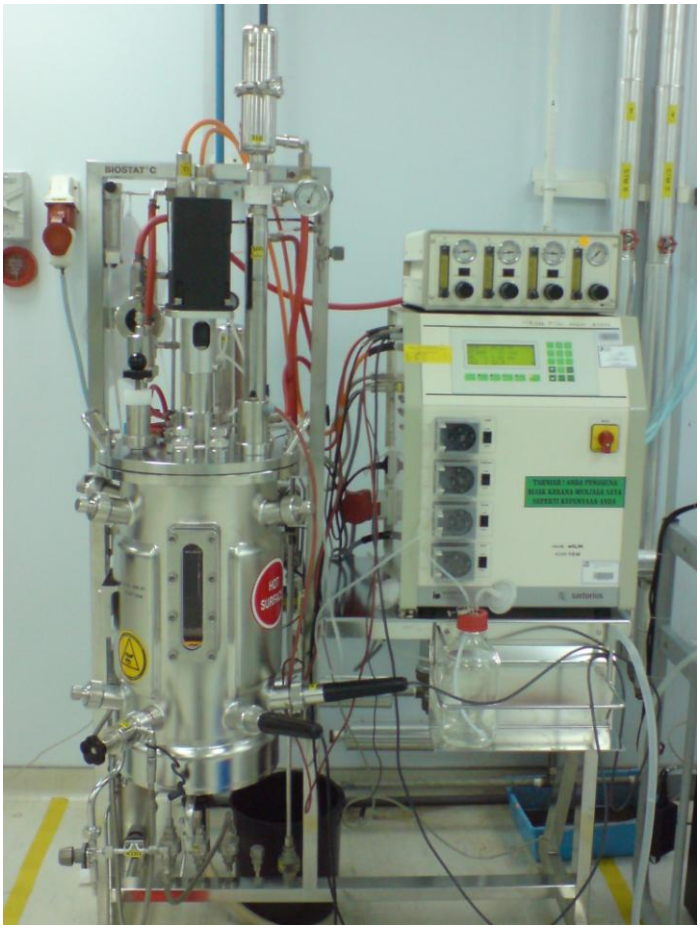

Figure B7: 20 L scale bioreactor system

Figure B8: Impeller and sparger in 20L bioreactor

APPENDIX C

RAW DATA FOR ALL EXPERIMENTS

Raw data for baseline data collection

Table C1: Cell in different basal media at 1% serum content

Type of medium	Maximum viable cell density (cells/mL)		Average	Standard Deviation
	1	2		
DMEM	10,000	13,000	11,500	2121
DF	800,000	1,100,000	950,000	212132
eRDF	1,150,000	1,250,000	1,200,000	70711

Table C2: Maximum viable cell density, maximum MAb titer and specific growth rate of hybridoma 192 culture in different media at different serum content

Type of medium	Maximum viable cell density		Maximum MAb titer		Specific growth rate	
	(cells/mL)	Standard deviation	(µg/mL)	Standard deviation	(1/h)	Standard deviation
Control (DMEM with 5% FBS)	1,383,125	304,940	0.700	0.021	0.032	0.003
DMEM with 2%FBS	1,196,042	65,402	0.488	0.349	0.034	0.002
DF with 0.5%FBS	580,782	69,605	0.037	0.001	0.022	0.002
eRDF with 0.5% FBS	885,625	225,390	0.034	0.001	0.027	0.004

Table C3: T-flask culture of hybridoma 192 in 0.5% DF

Average of Runs 1 and 2				
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**
0	211,200	185,000	87.6	0.000
24	360,625	280,625	77.8	0.008
48	645,938	531,563	82.3	0.019
72	744,375	466,875	62.7	0.030
96	678,750	67,500	9.9	0.036
Average of Runs 3 and 4				
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**
0	208,690	170,500	81.7	0.000
24	255,000	212,500	83.3	0.007
48	615,938	536,250	87.1	0.016
72	817,500	630,000	77.1	0.028
96	903,750	527,500	58.4	0.033
146.5	811,875	30,000	3.7	0.037

Table C4: T-flask culture of hybridoma 192 in 0.5% eRDF

Average of Runs 1 and 2				
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**
0	232,000	200,000	86.2	0.005
24	333,750	250,000	74.9	0.011
48	853,125	707,813	83.0	0.020
72	1,388,750	1,045,000	75.2	0.029
96	1,346,250	457,500	34.0	0.032
120	1,271,250	16,250	1.3	0.034
Average of Runs 3 and 4				
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**
0	218,300	198,000	90.7	0.002
24	416,250	382,500	91.9	0.010
48	813,750	658,750	81.0	0.017
72	981,250	726,250	74.0	0.026
96	922,500	65,000	7.0	0.033

Table C5: Glutamine concentration (mM) in different media at different incubation temperatures

Day	5% DMEM		2% DMEM		LSD	
	37°C	4°C	37°C	4°C	37°C	4°C
0	6.83	7.31	7.01	6.36	7.11	7.13
1	6.51	7.54	6.45	6.46	6.09	7.07
2	5.91	7.44	5.85	6.95	5.62	7.05
3	5.23	7.46	5.15	7.01	4.56	6.89
4	4.58	7.14	4.45	6.25	4.29	7.08
5	3.96	7.18	3.96	6.96	4.13	7.18
7	2.95	7.04	2.87	6.89	3.06	7.07
9	2.08	7.04	2.04	6.96	2.18	7.00
12	1.30	6.92	1.21	7.00	1.10	6.89
15	0.84	6.97	0.75	7.00	0.86	6.83
18	0.52	6.96	0.45	6.83	0.52	6.99
26	0.19	6.77	0.13	6.66	0.15	6.75
32	0.12	6.77	0.08	6.53	0.08	6.72
40	0.10	6.47	0.06	6.36	0.05	6.47
46	0.09	6.42	0.05	6.16	0.03	6.45
53	0.10	6.14	0.05	6.17	0.03	6.36
60	0.10	6.03	0.05	5.92	0.03	6.12

Table C6: T-flask culture of hybridoma 192 in 5% DMEM

Average of Runs 1 and 2								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	206,400	200,000	96.9	0.000	18.70	0.00	4.26	0.34
24	477,500	440,625	92.3	0.246	14.50	9.20	5.57	1.30
48	1,320,000	1,230,000	93.2	0.430	6.10	26.40	3.93	1.92
66	1,847,500	1,598,750	86.5	0.577	1.70	31.00	3.12	2.26
90	1,528,125	985,313	64.5	0.651	0.10	34.40	3.14	2.57
114	1,535,625	667,500	43.5	0.685	0.00	33.70	2.96	2.61
Average of Runs 3 and 4								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	207,470	200,000	96.4	0.000	18.70	0.00	4.26	0.34
24	329,375	305,625	92.8	0.333	15.80	7.20	5.85	1.26
48	701,250	660,000	94.1	0.539	8.60	18.60	4.43	1.71
66	1,295,000	1,167,500	90.2	0.622	3.60	25.40	3.79	2.13
90	1,260,000	967,500	76.8	0.711	0.50	28.10	3.51	2.43
114	1,093,125	564,375	51.6	0.715	0.00	28.60	3.47	2.52

Table C7: T-flask culture of hybridoma 192 in 2% DMEM

Average of Runs 1 and 2								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	213,900	200,000	93.5	0.000	24.40	0.00	3.39	0.43
23	245,000	124,375	50.8	0.203	18.00	5.60	3.37	1.51
47	497,500	363,750	73.1	0.304	12.90	13.50	2.16	1.80
71	1,153,125	968,438	84.0	0.365	6.60	26.00	0.91	2.34
95	1,640,000	1,253,750	76.4	0.373	2.10	32.20	0.27	2.84
119	1,871,875	621,875	33.2	0.353	0.30	33.90	0.16	3.06
141	1,859,375	50,000	2.7	0.379	0.10	33.50	0.14	3.04
Average of Runs 3 and 4								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	219,300	200,000	91.2	0.000	23.30	0.70	5.62	0.51
22	376,875	343,125	91.0	0.621	13.20	14.40	5.33	1.69
46	1,043,438	948,750	90.9	0.797	8.10	24.30	4.25	2.12
67	1,361,250	1,125,000	82.6	0.848	4.50	28.10	3.24	2.63
90	1,061,250	507,500	47.8	0.878	2.80	29.00	2.98	2.81
114	1,147,500	277,500	24.2	0.846	2.70	28.80	2.90	2.85
Average of Runs 5 and 6								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	230,950	200,000	86.6	0.076	23.90	1.30	3.48	0.39
23	503,125	440,625	87.6	0.070	15.00	15.00	3.33	1.40
29	669,375	582,188	87.0	0.087	13.20	17.35	3.27	1.62
47	1,193,750	1,035,000	86.7	0.156	7.35	24.25	2.92	2.37
53	1,328,125	1,120,313	84.4	0.157	6.10	25.70	2.80	2.69
71	1,590,000	1,209,375	76.1	0.188	3.20	27.50	2.54	3.46
77	1,479,375	939,375	63.5	0.162	2.35	27.30	2.46	3.58
96	1,460,000	490,000	33.6	0.207	2.05	28.55	2.46	3.78

Table C8: Comparison between T-flask cultures in 5% DMEM and 2% DMEM

Medium	Run	1	2	3	4	5	6	Average	Standard deviation
5% DMEM	μ	0.030	0.033	0.032	0.034	-	-	0.032	0.002
	r	0.0059	0.0056	0.0056	0.0059	-	-	0.0058	0.0002
	qGLU	0.284	0.335	0.209	0.308	-	-	0.284	0.054
	qLAC	0.525	0.508	0.455	0.498	-	-	0.497	0.030
	qGLN	0.034	0.018	0.005	0.017	-	-	0.019	0.010
	qAMM	0.040	0.039	0.030	0.050	-	-	0.040	0.008
2% DMEM	μ	0.045	0.035	0.032	0.032	0.032	0.038	0.036	0.005
	r	0.0033	0.0047	0.0061	0.0043	0.0024	0.0020	0.0038	0.0015
	qGLU	0.313		0.315		0.308		0.312	0.004
	qLAC	0.519		0.515		0.488		0.507	0.017
	qGLN	0.026		0.041		0.062		0.043	0.018
	qAMM	0.043		0.042		0.057		0.047	0.008

Key

μ	**Specific growth rate (1/h)**	**q**	**Specific consumption/production rate (mmol/10^6 cells.h)**
r	**Average specific MAb production rate (pg/cell.h)**	**GLU, LAC, GLN, AMM**	**Glucose, lactate, glutamine and ammonia, respectively**

Table C9: Spinner flask culture of hybridoma 192 in 5% DMEM

Average of Runs 1 and 2								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	241,250	216,250	89.6	0.177	20.50	3.75	5.51	0.56
22	307,500	254,375	82.7	0.248	18.10	6.40	5.40	0.97
28	396,500	336,250	84.8	0.248	16.45	8.05	5.26	1.16
46	1,024,688	945,938	92.3	0.273	9.75	16.65	4.51	2.19
52	1,365,000	1,292,813	94.7	0.278	7.85	18.60	3.97	2.42
70	2,397,500	2,257,500	94.2	0.279	4.30	21.95	1.98	4.10
76	2,568,750	2,248,438	87.5	0.280	2.90	22.50	1.28	3.66
96	2,521,875	553,125	21.9	0.257	1.15	24.45	1.31	5.36
Average of Runs 3 and 4								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	198,125	163,125	82.3	0.177	16.95	5.4	5.23	0.48
22	489,375	433,125	88.5	0.209	14.50	10.55	5.11	0.93
28	652,500	589,375	90.3	0.220	12.55	13.1	5.23	1.37
46	1,560,000	1,491,250	95.6	0.217	6.05	20.35	4.26	2.54
52	1,872,500	1,788,750	95.5	0.217	4.90	21.25	3.67	3.07
70	2,248,438	1,946,875	86.6	0.235	3.00	22.4	2.24	4.02
76	2,412,500	1,639,063	67.9	0.240	2.40	22.8	2.00	4.57
96	2,358,125	408,125	17.3	0.213	0.85	22.5	1.80	5.01

Table C9 (continue): Spinner flask culture of hybridoma 192 in 5% DMEM

Run 5								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	267,500	222,500	83.2	0.185	18.00	7.40	5.79	0.56
22	671,250	595,000	88.6	0.219	12.60	13.60	5.11	1.38
28	873,250	770,750	88.3	0.221	9.70	16.00	4.55	1.48
46	1,892,500	1,767,500	93.4	0.224	4.40	16.40	3.34	3.10
52	2,170,000	2,062,500	95.0	0.227	3.20	19.20	2.43	3.31
70	2,587,500	1,657,500	64.1	0.234	1.20	19.70	1.47	3.61
76	2,246,250	1,042,500	46.4	0.236	1.00	21.00	1.14	3.92
96	2,475,000	255,000	10.3	0.221	0.90	24.30	1.02	5.20
Run 6								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	241,250	225,000	93.3	0.205	19.50	5.00	5.68	0.56
19	330,000	256,250	77.7	0.242	17.50	7.90	5.19	0.86
25	382,500	313,750	82.0	0.247	16.20	10.00	5.15	1.08
43	942,500	865,000	91.8	0.271	9.00	18.50	4.49	1.72
49	1,407,500	1,285,000	91.3	0.278	6.80	23.20	4.10	2.34
67	2,118,750	2,002,500	94.5	0.279	3.20	28.80	2.19	3.94
73	2,242,500	2,010,000	89.6	0.262	2.40	29.20	1.70	4.33
92	2,610,000	660,000	25.3	0.263	0.90	24.50	1.15	4.34

Table C10: Spinner flask culture of hybridoma 192 in 2% DMEM (75 rpm)

Average of Runs 1 and 2								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	200,000	200,000	93.2	0.000	23.10	0.70	5.39	0.00
16.5	441,875	423,750	95.9	0.025	16.50	11.60	4.96	16.50
23.5	526,875	485,625	92.2	0.033	14.50	14.45	4.86	23.50
40.5	1,067,813	1,011,563	94.7	0.068	7.90	24.70	3.65	40.50
47	1,600,000	1,471,250	92.0	0.081	5.90	24.75	2.78	47.00
65	1,826,563	1,284,375	70.3	0.138	3.85	29.80	1.74	65.00
89	1,838,750	232,500	12.6	0.134	1.35	31.00	1.60	89.00
Average of Runs 3 and 4								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	265,000	215,625	81.4	0.231	19.90	6.75	5.68	0.83
22	503,438	398,438	79.1	0.285	14.95	12.55	5.23	1.41
28	654,375	543,750	83.1	0.299	12.95	15.40	5.07	2.28
46	1,308,000	1,185,000	90.6	0.323	6.95	24.70	4.16	2.94
52	1,455,000	1,310,000	90.0	0.328	5.90	26.45	3.63	3.40
70	1,981,250	1,381,250	69.7	0.344	3.70	29.20	2.42	5.03
94	1,843,750	167,188	9.1	0.293	1.30	30.40	2.17	5.34

Table C10 (continue): Spinner flask culture of hybridoma 192 in 2% DMEM (75 rpm)

Run 5								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	252,500	230,000	91.1	0.060	19.80	4.80	5.33	0.70
19	518,750	465,000	89.6	0.069	16.40	10.80	5.11	1.45
25	562,500	507,500	90.2	0.077	14.50	13.40	4.79	1.57
43	1,365,000	1,297,500	95.1	0.099	7.80	23.50	3.57	2.79
49	1,487,500	1,330,000	89.4	0.106	5.50	27.10	2.85	3.42
67	1,650,000	1,050,000	63.6	0.141	2.40	25.70	1.50	3.87
73	2,077,500	1,005,000	48.4	0.146	1.40	24.50	1.34	4.04
Run 6								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	241,250	226,250	93.8	0.000	20.30	4.50	4.99	0.63
19	393,750	355,000	90.2	0.001	15.40	8.80	4.65	1.13
25	502,500	450,000	89.6	0.002	15.20	12.50	4.81	1.57
43	1,461,500	1,390,000	95.1	0.028	7.10	23.20	3.52	2.75
49	1,452,500	1,342,500	92.4	0.033	5.40	24.00	2.61	2.61
67	1,683,750	1,053,750	62.6	0.053	3.10	29.00	1.59	4.40
73	1,860,000	975,000	52.4	0.109	2.00	28.50	1.44	4.65

Table C11: Comparison between spinner flask cultures in 5% DMEM and 2% DMEM (75 rpm)

Medium	Run	1	2	3	4	5	6	Average	Standard deviation
5% DMEM	μ	0.048	0.049	0.049	0.044	0.043	0.046	0.047	0.003
	r	0.0019	0.0019	0.0007	0.0009	0.0006	0.0015	0.0013	0.0006
	qGLU	0.199	0.199	0.171	0.146	0.171	0.209	0.183	0.024
	qLAC	0.212	0.224	0.218	0.191	0.171	0.385	0.234	0.077
	qGLN	0.043	0.042	0.034	0.032	0.042	0.050	0.041	0.006
	qAMM	0.047	0.053	0.044	0.046	0.046	0.043	0.046	0.003
2% DMEM	μ	0.041	0.040	0.038	0.032	0.055	0.041	0.041	0.008
	r	0.0033	0.0047	0.0061	0.0043	0.0024	0.0020	0.0038	0.0015
	qGLU	0.304	0.308	0.239	0.261	0.302	0.306	0.287	0.029
	qLAC	0.436	0.437	0.312	0.342	0.393	0.464	0.397	0.060
	qGLN	0.052	0.052	0.044	0.048	0.064	0.058	0.053	0.007
	qAMM	0.072	0.073	0.060	0.063	0.056	0.068	0.065	0.007

Key

μ **Specific growth rate (1/h)**

r **Average specific MAb production rate (pg/cell.h)**

q **Specific consumption/production rate (mmol/10^6 cells.h)**

GLU, LAC, GLN, AMM **Glucose, lactate, glutamine and ammonia, respectively**

Table C12: Bioreactor culture of hybridoma 192 in 2% DMEM

Run 1								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	163,125	146,250	89.7	0.057	24.42	5.44	3.69	1.35
18	280,313	264,375	94.3	0.072	22.10	7.86	3.58	2.28
22	362,813	333,750	92.0	0.076	21.92	9.08	3.27	2.50
41	433,750	405,000	93.4	0.143	17.81	16.06	1.52	3.43
44	550,000	503,750	91.6	0.141	15.71	17.00	2.11	3.69
48	570,000	515,000	90.4	0.169	13.01	17.81	1.92	3.80
66	781,875	553,125	70.7	0.298	8.82	24.56	0.83	4.57
70	825,000	538,125	65.2	0.306	8.11	24.92	0.56	5.06
90	790,000	265,000	33.5	0.442	5.75	29.03	0.27	5.32
Run 2								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	309,375	270,000	87.3	0.033	22.30	5.11	4.45	1.10
18	316,875	263,438	83.1	0.039	19.80	8.62	4.1	1.70
24	434,063	351,563	81.0	0.038	17.28	10.36	4.06	2.32
43	768,750	658,750	85.7	0.055	10.00	19.10	2.08	3.59
45	826,250	701,250	84.9	0.053	8.96	20.34	2.37	3.67
47	892,500	800,000	89.6	0.064	7.98	20.73	1.75	3.72
66	1,170,000	896,250	76.6	0.130	2.33	31.23	0.85	4.29
72	1,151,250	581,250	50.5	0.140	1.87	32.01	0.55	5.31
92	1,226,250	123,750	10.1	0.138	1.78	32.70	0.25	5.27
Run 3								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	219,375	211,875	96.6	0.054	22.21	4.53	4.35	2.34
18	420,000	392,500	93.5	0.067	19.27	7.90	4.09	3.23
24	558,750	533,750	95.5	0.073	17.87	10.56	3.65	3.40
43	847,500	805,000	95.0	0.114	10.06	20.15	2.61	4.60
46	1,025,000	967,500	94.4	0.114	7.96	22.44	1.89	4.76
49	1,167,500	1,065,000	91.2	0.138	6.28	26.42	1.22	4.86
67	1,357,500	622,500	45.9	0.257	2.06	33.30	0.45	5.29
69	1,567,500	528,750	33.7	0.244	1.80	32.84	0.11	5.19
92	1,456,875	39,375	2.7	0.277	2.29	32.17	0.08	4.31

Table C13: Specific growth rate, average specific MAb production rate and metabolites consumption / production rates of bioreactor culture in 2% DMEM

Run	1	2	3	Average	Standard deviation
μ	0.027	0.037	0.033	0.032	0.005
r	0.0109	0.0023	0.0045	0.0059	0.0045
qGLU	0.528	0.446	0.406	0.460	0.062
qLAC	0.689	0.611	0.578	0.626	0.057
qGLN	0.097	0.090	0.086	0.091	0.006
qAMM	0.118	0.093	0.045	0.085	0.037

See Table C11 for an explanation of symbols and units

Table C14: T-flask culture of hybridoma 192 in 2% DMEM (current stock)

Average of runs 1 and 2								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (μg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	228,750	214,688	93.9	0.064	29.44	4.85	4.29	1.66
23	332,500	278,750	83.7	0.109	22.44	9.54	3.27	2.12
30	519,375	445,000	85.6	0.121	21.63	12.55	2.62	2.53
47	932,500	810,000	86.9	0.136	15.10	24.53	2.06	3.14
54	1,548,750	1,451,250	93.7	0.154	10.18	26.28	1.87	3.41
71	2,233,438	1,892,188	84.7	0.215	5.57	34.64	1.36	5.12
78	2,047,500	1,590,000	77.6	0.220	4.17	37.58	0.70	5.20
95	1,960,000	717,500	36.6	0.260	3.28	44.04	0.31	6.03
Average of runs 3 and 4								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (μg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	253,125	217,500	85.9	0.050	23.07	4.51	5.00	0.95
18	326,875	274,375	83.9	0.104	19.88	7.45	4.78	1.67
25	371,250	313,125	84.4	0.135	18.67	9.76	4.03	2.00
41	885,000	803,750	90.8	0.221	13.21	17.00	3.30	2.77
45	1,058,750	943,750	89.1	0.214	12.27	19.03	2.70	2.92
49	1,320,000	1,216,250	92.1	0.307	10.88	21.92	1.89	3.20
66	1,644,375	1,314,375	79.9	0.362	4.96	28.56	1.74	4.20
73	1,828,125	1,318,125	72.1	0.385	3.94	29.45	1.07	4.51
96	1,932,500	282,500	14.6	0.431	2.24	30.75	0.63	4.78

Table C15: Specific growth rate, average specific MAb production rate and specific metabolites consumption / production rates of hybridoma 192 in T-flask culture using 2% DMEM (current stock)

Run	1	2	3	4	Average	Standard deviation
μ	0.048	0.041	0.047	0.046	0.045	0.003
r	0.0026	0.0024	0.0062	0.0059	0.0043	0.0021
qGLU	0.326		0.322		0.324	0.003
qLAC	0.510		0.399		0.455	0.078
qGLN	0.049		0.067		0.058	0.013
qAMM	0.057		0.058		0.058	0.000

See Table C11 for an explanation of symbols and units

Table C16: Fed-batch culture of hybridoma 192 in 5% DMEM (40 rpm)

Run 1								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (μg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	210,000	191,250	91.1	0.379	18.52	3.96	6.39	0.89
24	461,250	438,750	95.1	0.531	14.91	10.33	6.02	1.61
30	650,000	625,000	96.2	0.528	12.89	14.15	4.59	2.14
48	1,815,000	1,777,500	97.9	0.553	5.44	25.52	3.00	3.29
49	792,500	776,250	97.9	0.557	15.85	10.15	5.95	1.50
54	855,000	836,250	97.8	0.550	15.13	12.63	4.47	1.51
72	1,700,000	1,681,250	98.9	0.595	6.83	23.89	3.87	3.00
78	2,037,500	2,006,250	98.5	0.601	5.78	28.00	3.28	3.06
96	2,287,500	2,156,250	94.3	0.563	3.67	33.44	2.42	3.97

Table C16 (continue): Fed-batch culture of hybridoma 192 in 5% DMEM (40 rpm)

Run 2								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	202,500	190,000	93.8	0.375	18.83	4.04	8.57	0.72
24	465,000	440,000	94.6	0.524	15.94	11.00	6.97	1.89
30	731,250	711,250	97.3	0.492	13.28	14.52	5.37	1.99
48	1,800,000	1,775,000	98.6	0.549	5.69	27.41	3.12	3.78
49	788,750	761,250	96.5	0.521	16.98	10.48	6.87	1.60
54	810,000	793,125	97.9	0.546	15.91	12.22	4.84	1.61
72	1,778,125	1,750,000	98.4	0.574	7.35	27.78	4.04	2.86
78	2,062,500	2,012,500	97.6	0.596	5.19	28.67	3.54	3.08
96	2,133,750	2,013,750	94.4	0.556	3.67	31.31	2.59	3.26
Run 3								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	243,750	230,000	94.4	0.047	19.99	5.64	6.05	0.42
20	573,750	542,500	94.6	0.056	13.36	11.94	5.94	1.57
26	853,750	830,000	97.2	0.056	12.13	17.19	4.49	1.94
43.5	1,952,500	1,912,500	98.0	0.082	5.31	25.19	3.03	3.05
44.5	451,250	441,250	97.8	0.046	14.99	9.53	5.29	0.32
50	748,125	727,500	97.2	0.067	13.40	10.92	3.68	1.58
68	1,546,875	1,521,875	98.4	0.102	7.47	26.36	3.63	1.87
74	1,830,000	1,811,250	99.0	0.108	5.43	27.56	2.86	2.93
92	1,898,750	1,789,375	94.2	0.129	3.60	31.36	2.57	3.81

Table C17: Fed-batch culture of hybridoma 192 in 2% DMEM (40 rpm)

Run 1								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	185,000	151,250	81.8	0.055	18.42	5.36	7.18	1.05
22.5	245,000	210,000	85.7	0.110	16.81	9.06	6.37	1.24
28.5	355,000	317,500	89.4	0.135	14.50	10.64	5.02	1.87
46.5	880,000	812,500	92.3	0.236	9.44	21.28	3.54	2.51
52.5	1,180,000	1,117,500	94.7	0.280	6.83	23.72	1.99	3.12
53.5	358,750	333,750	93.0	0.135	16.46	9.08	5.02	1.63
70.5	745,000	710,000	95.3	0.239	8.10	12.81	4.09	1.73
74.5	957,500	912,500	95.3	0.255	9.54	20.75	3.79	2.21
77.5	1,020,000	990,000	97.1	0.204	8.86	23.67	3.66	2.22
94.5	1,376,250	1,233,750	89.6	0.257	3.65	29.92	2.78	3.56
100.5	1,132,500	933,750	82.5	0.289	2.83	28.58	2.49	3.65
Run 2								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	213,750	176,250	82.5	0.062	17.35	4.89	6.52	1.02
22.5	231,250	186,250	80.5	0.120	16.35	8.50	5.90	1.70
28.5	332,500	268,750	80.8	0.154	15.64	10.61	5.11	2.18
46.5	780,000	717,500	92.0	0.259	10.38	21.08	4.37	2.73
52.5	1,162,500	1,075,000	92.5	0.300	7.31	24.50	3.93	3.16
53.5	416,250	385,000	92.5	0.166	15.43	8.14	4.65	1.93
70.5	767,500	727,500	94.8	0.270	12.47	18.17	3.80	2.11
74.5	950,000	900,000	94.7	0.297	9.71	19.28	3.37	2.13
77.5	1,045,000	992,500	95.0	0.274	8.89	22.15	3.12	2.75
94.5	1,361,250	1,207,500	88.7	0.317	3.35	30.58	2.35	3.41
100.5	1,372,500	1,188,750	86.6	0.303	2.75	31.89	2.44	3.71

Table C17 (continue): Fed-batch culture of hybridoma 192 in 2% DMEM (40 rpm)

Run 3								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	161,250	141,250	87.6	0.043	19.47	3.67	7.18	0.62
20	396,250	377,500	95.3	0.050	17.19	8.64	6.67	0.85
26	520,000	483,750	93.0	0.043	15.22	11.22	6.39	1.07
43.5	1,242,500	1,205,000	97.0	0.046	8.58	22.19	4.58	1.47
44.5	375,000	366,250	97.7	0.042	17.25	8.44	7.25	0.94
50	545,625	536,250	98.3	0.063	16.11	10.25	6.96	1.46
68	1,290,625	1,268,750	98.3	0.072	10.11	21.14	3.95	1.55
74	1,665,000	1,646,250	98.9	0.086	7.74	23.14	2.72	2.67
92	1,929,375	1,710,625	88.7	0.066	4.75	27.25	1.79	3.51

Raw data for medium screening and optimization

Table C18: Experimental design layout and the data of responses for screening and optimization

Run	AA (%)	2-M (µL/L)	FeC (µM)	EtA (µL/L)	NaSe (nM)	$ZnSO_4$ (µM)	Nv (cells/mL)	%V (%)	p (µg/mL)	r (pg/cell.h)
1	0.00	0.00	0.00	3.00	25.00	0.00	177069	34.1	0.140	3.04E-08
2	0.00	0.00	0.00	0.00	25.00	6.00	835764	79.3	0.202	9.30E-09
3	2.00	4.00	0.00	3.00	25.00	0.00	172347	27.5	0.169	3.77E-08
4	0.00	4.00	0.00	3.00	0.00	6.00	144016	23.3	0.177	4.72E-08
5	2.00	0.00	500.00	3.00	25.00	0.00	373025	47.5	0.182	1.87E-08
6	2.00	4.00	500.00	3.00	0.00	6.00	779102	65.2	0.230	1.14E-08
7	0.00	0.00	500.00	3.00	0.00	6.00	1128520	88.0	0.219	7.47E-09
8	0.00	4.00	500.00	0.00	25.00	6.00	1662090	98.1	0.164	3.79E-09
9	0.00	0.00	500.00	0.00	0.00	0.00	519401	51.5	0.148	1.10E-08
10	2.00	0.00	500.00	0.00	25.00	6.00	1775410	97.0	0.204	4.42E-09
11	2.00	0.00	0.00	0.00	0.00	0.00	283310	38.8	0.144	1.96E-08
12	2.00	4.00	0.00	0.00	25.00	6.00	236092	31.5	0.155	2.53E-08
13	1.00	2.00	250.00	1.50	12.50	3.00	1629030	85.5	0.263	6.20E-09
14	0.00	4.00	500.00	3.00	25.00	0.00	439130	47.5	0.150	1.32E-08
15	0.00	4.00	0.00	0.00	0.00	0.00	127489	19.0	0.119	3.60E-08
16	2.00	0.00	0.00	3.00	0.00	6.00	505236	59.1	0.166	1.27E-08
17	2.00	4.00	500.00	0.00	0.00	0.00	396634	45.8	0.096	9.31E-09
18	2.00	4.00	0.00	3.00	0.00	0.00	149554	23.2	0.262	6.73E-08
19	2.00	0.00	0.00	3.00	25.00	6.00	448662	50.3	0.306	2.63E-08
20	0.00	4.00	500.00	3.00	0.00	0.00	350517	39.7	0.254	2.78E-08
21	1.00	2.00	250.00	1.50	12.50	3.00	754001	66.4	0.289	1.47E-08
22	2.00	0.00	500.00	0.00	0.00	6.00	1121650	82.1	0.263	9.03E-09
23	0.00	0.00	500.00	0.00	25.00	0.00	631450	52.1	0.244	1.48E-08
24	2.00	4.00	0.00	0.00	0.00	6.00	299108	36.8	0.202	2.60E-08
25	0.00	0.00	0.00	3.00	0.00	0.00	267951	37.7	0.253	3.63E-08
26	2.00	4.00	500.00	3.00	25.00	6.00	1134120	64.1	0.299	1.02E-08
27	0.00	4.00	0.00	0.00	25.00	0.00	130860	20.1	0.279	8.19E-08
28	2.00	0.00	0.00	0.00	25.00	0.00	272105	39.2	0.255	3.60E-08
29	0.00	4.00	500.00	0.00	0.00	6.00	1218240	71.7	0.251	7.94E-09
30	2.00	0.00	500.00	3.00	0.00	0.00	311571	39.3	0.239	2.95E-08

Table C18 (continue): Experimental design layout and the data of responses for screening and optimization

Run	AA (%)	2-M (µL/L)	FeC (µM)	EtA (µL/L)	NaSe (nM)	$ZnSO_4$ (µM)	Nv (cells/mL)	%V (%)	p (µg/mL)	r (pg/cell.h)
31	0.00	0.00	0.00	0.00	0.00	6.00	545249	64.6	0.221	1.56E-08
32	0.00	0.00	500.00	3.00	25.00	6.00	495397	57.0	0.212	1.64E-08
33	0.00	4.00	0.00	3.00	25.00	6.00	328188	43.0	0.208	2.44E-08
34	2.00	4.00	500.00	0.00	25.00	0.00	414389	39.1	0.198	1.84E-08
35	2.00	4.00	0.00	3.00	25.00	0.00	86883	13.9	0.185	8.18E-08
36	0.00	0.00	0.00	0.00	25.00	6.00	566240	50.2	0.212	1.44E-08
37	2.00	0.00	0.00	3.00	0.00	6.00	362513	40.6	0.206	2.19E-08
38	0.00	4.00	0.00	0.00	0.00	0.00	71903	10.1	0.168	9.00E-08
39	2.00	0.00	0.00	0.00	0.00	0.00	257654	34.3	0.196	2.93E-08
40	2.00	0.00	500.00	3.00	25.00	0.00	458385	44.1	0.228	1.91E-08
41	0.00	4.00	500.00	3.00	25.00	0.00	420186	38.2	0.172	1.57E-08
42	2.00	4.00	500.00	3.00	0.00	6.00	801424	64.8	0.157	7.56E-09
43	1.00	2.00	250.00	1.50	12.50	3.00	2102430	79.4	0.225	4.11E-09
44	2.00	0.00	500.00	0.00	25.00	6.00	1174420	82.6	0.212	6.94E-09
45	0.00	4.00	0.00	3.00	0.00	6.00	60669	10.9	0.147	9.33E-08
46	0.00	0.00	0.00	3.00	25.00	0.00	182006	30.8	0.189	3.99E-08
47	0.00	0.00	500.00	0.00	0.00	0.00	581969	48.9	0.181	1.20E-08
48	0.00	0.00	500.00	3.00	0.00	6.00	1022380	78.0	0.210	7.92E-09
49	2.00	4.00	500.00	0.00	0.00	0.00	260650	31.2	0.121	1.78E-08
50	2.00	4.00	0.00	0.00	25.00	6.00	98867	14.3	0.153	5.97E-08
51	0.00	4.00	500.00	0.00	25.00	6.00	772962	64.5	0.171	8.50E-09
52	0.00	0.00	0.00	0.00	0.00	6.00	438909	47.0	0.195	1.71E-08
53	2.00	4.00	0.00	0.00	0.00	6.00	131291	13.3	0.146	4.28E-08
54	0.00	0.00	0.00	3.00	0.00	0.00	329754	34.3	0.179	2.09E-08
55	0.00	0.00	500.00	0.00	25.00	0.00	551880	51.3	0.172	1.20E-08
56	2.00	0.00	500.00	3.00	0.00	0.00	558750	42.5	0.181	1.24E-08
57	0.00	0.00	500.00	3.00	25.00	6.00	343494	37.8	0.193	2.16E-08
58	2.00	4.00	500.00	3.00	25.00	6.00	708361	61.9	0.179	9.72E-09
59	2.00	0.00	0.00	3.00	25.00	6.00	581650	46.4	0.188	1.24E-08
60	2.00	4.00	0.00	3.00	0.00	0.00	91598	10.9	0.169	7.10E-08
61	2.00	4.00	500.00	0.00	25.00	0.00	444252	37.6	0.136	1.18E-08
62	0.00	4.00	500.00	0.00	0.00	6.00	570200	50.4	0.166	1.12E-08

Table C18 (continue): Experimental design layout and the data of responses for screening and optimization

Run	AA (%)	2-M (µL/L)	FeC (µM)	EtA (µL/L)	NaSe (nM)	$ZnSO_4$ (µM)	Nv (cells/mL)	%V (%)	p (µg/mL)	r (pg/cell.h)
63	2.00	0.00	500.00	0.00	0.00	6.00	1001480	66.4	0.183	7.04E-09
64	2.00	0.00	0.00	0.00	25.00	0.00	619816	47.8	0.202	1.25E-08
65	1.00	2.00	250.00	1.50	12.50	3.00	1181620	68.5	0.211	6.86E-09
66	0.00	4.00	0.00	0.00	25.00	0.00	283955	22.4	0.152	2.06E-08
67	0.00	4.00	0.00	3.00	25.00	6.00	190830	21.0	0.183	3.69E-08
68	0.00	4.00	500.00	3.00	0.00	0.00	364103	35.4	0.185	1.95E-08
69	1.00	2.00	250.00	1.50	12.50	3.00	717279	70.7	0.300	1.61E-08
70	1.00	2.00	250.00	1.50	12.50	3.00	801481	75.6	0.340	1.63E-08
71	1.00	2.00	250.00	1.50	12.50	3.00	804600	73.2	0.330	1.58E-08
72	1.00	2.00	250.00	1.50	12.50	3.00	1079040	77.5	0.317	1.13E-08
73	1.00	2.00	0.00	1.50	12.50	3.00	166807	32.0	0.135	3.11E-08
74	1.00	2.00	250.00	1.50	12.50	3.00	765896	69.2	0.196	9.83E-09
75	1.00	2.00	500.00	1.50	12.50	3.00	869470	69.8	0.242	1.07E-08
76	1.00	2.00	250.00	1.50	12.50	3.00	801329	70.3	0.257	1.24E-08
77	2.00	2.00	250.00	1.50	12.50	3.00	962140	77.5	0.249	9.97E-09
78	0.00	2.00	250.00	1.50	12.50	3.00	787701	69.1	0.245	1.20E-08
79	1.00	2.00	250.00	1.50	12.50	6.00	910354	73.2	0.249	1.05E-08
80	1.00	2.00	250.00	1.50	12.50	3.00	809506	70.7	0.187	8.88E-09
81	1.00	2.00	250.00	1.50	12.50	3.00	695030	67.7	0.196	1.08E-08
82	1.00	2.00	250.00	1.50	12.50	3.00	738640	68.4	0.200	1.04E-08
83	1.00	2.00	250.00	1.50	25.00	3.00	880372	76.0	0.250	1.09E-08
84	1.00	2.00	250.00	3.00	12.50	3.00	812232	63.8	0.242	1.14E-08
85	1.00	2.00	250.00	1.50	0.00	3.00	550573	58.9	0.225	1.57E-08
86	1.00	0.00	250.00	1.50	12.50	3.00	782250	76.1	0.268	1.32E-08
87	1.00	2.00	250.00	1.50	12.50	3.00	825860	69.7	0.252	1.17E-08
88	1.00	2.00	250.00	0.00	12.50	3.00	545122	66.9	0.162	1.14E-08
89	1.00	4.00	250.00	1.50	12.50	3.00	749543	68.9	0.162	8.32E-09
90	1.00	2.00	250.00	1.50	12.50	0.00	287824	41.9	0.190	2.54E-08

Key for Table C18

□ & ▭	-	represent different blocks	$ZnSO_4$	-	Zinc sulfate
AA	-	Amino acids	Nv	-	Maximum viable cell density
2-M	-	2-Mercaptoethanol	%V	-	% Viability
FeC	-	Ferric citrate	p	-	Maximum MAb titer
EtA	-	Ethanolamine	r	-	Average specific MAb production rate
NaSe	-	Sodium selenite			

Table C19: Adaptation of hybridoma 192 in NM + 0.4% serum

Time (h)	Viable cell density (cells/mL)	% Viability
0	238,000	96
24	600,000	95.2
48	715,625	92
48	250,000	92
72	490,000	90.3
96	703,125	89.3
96	250,000	89.3
120	403,125	84.0
144	700,000	75.4
144	220,000	75.4
168	301,250	78.2
180	421,250	88.5
180	200,000	88.5
198	211,250	71.9
216	249,375	77.3
216	200,000	77.3
240	341,250	74.2
264	521,250	72.4
264	200,000	72.4
288	307,500	74.1
312	639,375	78.6
312	200,000	78.6
336	276,250	77.8
360	714,375	86.0

Table C20: Adaptation of hybridoma 192 in NM + 0.1% serum

Serum content (%)	Time (h)	Viable cell density (cells/mL)	% Viability
0.4	0	200,000	95.9
	24	467,500	96.4
	48	841,875	89.6
	48	200,000	89.6
	72	386,250	86.6
	96	738,750	87
	96	200,000	87
	120	392,500	86.5
	144	849,375	90.6
	144	200,000	90.6
	168	341,250	89.5
	192	813,750	91.9
0.1	192	200,000	91.9
	216	285,000	92.7
	238	483,750	90.5
	238	200,000	90.5
	262	256,250	84.7
	286	248,750	72.1
	286	200,000	72.1
	334	236,250	51.9
	334	165,200	51.9
	382	160,000	35.5

Table C21: T-flask culture of hybridoma 192 in LSD

Run 1								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	204,375	197,813	96.8	0.066	17.60	3.49	6.19	0.88
23	371,250	325,000	87.5	0.110	16.20	7.19	5.93	1.13
26	376,250	328,750	87.4	0.113	15.75	7.83	5.74	1.21
29	380,000	330,000	86.8	0.131	15.45	9.48	5.52	1.23
46	721,875	626,250	86.8	0.205	11.60	15.30	4.26	1.41
49	733,125	650,625	88.7	0.221	10.45	18.95	4.18	1.67
52	802,500	721,875	90.0	0.247	9.93	22.10	4.06	2.04
70	1,568,750	1,390,625	88.6	0.899	4.24	31.85	1.93	4.13
74	1,803,125	1,506,250	83.5	0.937	3.40	33.80	1.37	4.22
95	1,566,250	74,375	4.7	1.091	0.63	34.55	0.98	5.55
Run 2								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	233,438	215,625	92.4	0.065	19.30	3.62	7.10	0.85
23	357,500	308,750	86.4	0.107	16.75	8.23	6.78	1.27
26	407,500	346,250	85.0	0.114	16.20	8.89	6.48	1.31
29	410,000	356,250	86.9	0.133	15.70	10.30	6.16	1.52
46	648,750	560,625	86.4	0.202	11.30	18.30	4.76	1.88
49	695,625	588,750	84.6	0.209	10.55	19.00	4.66	2.10
52	890,625	793,125	89.1	0.234	9.09	19.75	4.08	2.79
70	1,640,625	1,462,500	89.1	0.735	4.04	28.75	1.96	3.30
74	1,759,375	1,509,375	85.8	0.929	3.56	30.50	1.59	4.18
95	1,701,875	126,875	7.5	1.264	1.00	30.90	0.85	5.37

Table C21 (continue): T-flask culture of hybridoma 192 in LSD

Run 3								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	213,750	195,000	91.2	0.259	19.95	3.47	5.85	3.41
24	457,500	432,500	94.5	0.525	16.90	9.52	5.18	4.33
28	533,750	498,750	93.4	0.603	16.25	11.55	4.6	4.57
31	560,000	516,250	92.2	0.652	15.35	13.00	4.25	4.84
47	1,015,000	942,500	92.9	1.383	8.97	24.00	3.27	5.80
51	1,327,500	1,237,500	93.2	1.453	7.49	26.80	2.94	6.17
55	1,357,500	1,222,500	90.1	1.681	6.18	28.50	2.52	6.43
72	1,796,250	1,428,750	79.5	6.277	3.00	32.20	1.41	7.32
78	1,957,500	1,110,000	56.7	7.443	2.42	33.35	1.28	7.94
96	1,815,000	5,000	0.3	7.899	1.42	33.00	1.22	8.10
Run 4								
Time (h)	**Total cells (cells/mL)**	**Viable cells (cells/mL)**	**% viability**	**MAb titer (µg/mL)**	**Glucose conc. (mM)**	**Lactate conc. (mM)**	**Glutamine conc. (mM)**	**Ammonia conc. (mM)**
0	199,688	185,625	93.0	0.243	19.00	3.11	5.26	4.12
24	452,500	430,000	95.0	0.515	17.25	8.96	4.76	4.91
28	486,250	451,250	92.8	0.537	16.75	10.85	4.72	5.4
31	531,250	497,500	93.6	0.611	15.75	12.10	4.50	5.57
47	952,500	890,000	93.4	1.188	9.88	21.95	3.50	6.22
51	1,035,000	960,000	92.8	1.225	8.76	25.35	3.32	6.68
55	1,197,500	1,117,500	93.3	1.453	6.86	26.25	2.83	6.8
72	1,725,000	1,530,000	88.7	5.265	3.30	31.60	1.55	7.34
78	1,980,000	1,275,000	64.4	5.966	2.61	32.05	1.33	7.53
96	1,650,000	50,000	3.0	7.491	1.42	32.50	1.21	7.39

Table C22: Specific growth rate, average specific MAb production rate and specific metabolites consumption / production rates for T-flask culture of hybridoma 192 in LSD

Run	1	2	3	4	Average	Standard Deviation
μ	0.034	0.032	0.034	0.032	0.033	0.001
r	0.0169	0.0187	0.0993	0.0928	0.0569	0.0453
qGLU	0.276	0.255	0.273	0.252	0.264	0.012
qGLN	0.084	0.084	0.068	0.056	0.073	0.014
qLAC	0.581	0.433	0.495	0.489	0.500	0.061
qAMM	0.077	0.076	0.077	0.060	0.073	0.008
Max viable cell density (cells/mL)	1,302,500	1,541,250	1,428,750	1,530,000	1,450,625	110,955
Max MAb titer (μg/mL)	1.091	1.264	7.899	7.491	4.436	3.767

Key

μ	Specific growth rate (1/h)	q	Specific consumption/production rate (mmol/10^6 cells.h)
r	Average specific MAb production rate (pg/cell.h)	GLU, LAC, GLN, AMM	Glucose, lactate, glutamine and ammonia, respectively

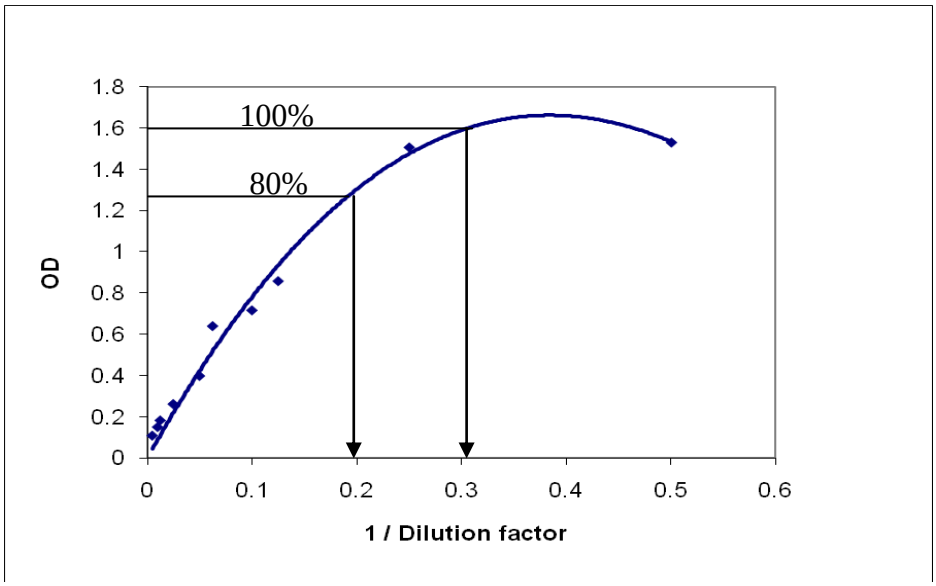

Figure C1: Optical density of purified MAb from T-flask culture in LSD versus inverse of dilution factor

Table C23: Cross-reactivity of MAb with various hormones

Concentration of hormones (ng/mL)	Optical density				
	17-OHP	**PREG17**	**PREG5**	**ANDRD**	**BESTR**
0	0.7772	0.8732	0.8000	0.8000	0.8407
25	0.8587	0.8577	0.7410	0.6620	0.9862
50	0.7387	0.9657	0.6595	0.6685	0.9897
100	0.6537	0.7702	0.6945	0.7610	0.8102
250	0.5952	0.8587	0.7255	0.9575	0.7447
500	0.4162	0.8797	0.7780	0.9205	1.0737
1000	0.1452	1.1527	0.7505	0.9895	1.1147
1500	0.1477	0.8192	0.4625	0.7160	0.9127
2000	0.0282	0.8152	0.4445	-	-

Key

17-OHP - 17α-hydroxyprogesterone

PREG17 - 17α-hydroxypregnenolone

PREG5 - 5-pregnen-3β-ol-20-one

ANDRD - 4-androstene-3,17-dione

BESTR - β-estradiol

Table C24: Price of components used in the culture medium

Component of culture medium	Price in RM* (2008)	Amount used per L	Price in RM per L
DMEM	120 / 10 L	1	12.0
L-glutamine	394.5 / 100 g	0.585 g	2.3
Sodium bicarbonate	142.5 / 500 g	3.7 g	1.1
Streptomycin - penicillin	49 / 20 mL (100 x)	10 mL	0.25
Fetal bovine serum	2913 / 500 mL **	50 mL (5%) 20 mL (2%) 4 mL (0.4%)	291.3 116.5 23.3
Ferric citrate	218 / 250 g	0.076 g	0.07
Zinc sulfate	132 / 100 g	0.001 g	< 0.01
Sodium selenite	100.5 / 10 g	0.000003 g	< 0.00
Total cost per L of DMEM with 5% serum (RM)		12 + 2.3 + 1.1 + 0.25 + 291.3 = 306.95	
Total cost per L of DMEM with 2% serum (RM)		12 + 2.3 + 1.1 + 0.25 + 116.5 = 132.15	
Total cost per L of LSD with 0.4% serum (RM)		12 + 2.3 + 1.1+ 0.25 + 23.3 + 0.07 + 0.01 + 0.00 = 39.03	

* RM is Malaysian ringgit; ** Price quoted in year 2007

Table C25: Percentage of cost reduction between media

Comparison between	Percentage of cost reduction
DMEM with 5% serum and 2% serum	$\frac{306.95-132.15}{306.95}\times 100\% = 56.9\%$
DMEM with 2% serum and LSD	$\frac{132.15-39.03}{132.15}\times 100\% = 70.5\%$
DMEM with 5% and LSD	$\frac{306.95-39.03}{306.95}\times 100\% = 87.3\%$

Raw data for inducer screening and optimization

Table C26: Results of ELISA for screening experiments

Amino acids (%)	1	2	3	Average	Standard Deviation
0	0.513	0.820	-	0.667	0.217
1	0.373	0.478	-	0.425	0.065
2	0.477	0.871	-	0.674	0.309
3	0.331	0.588	-	0.459	0.123
4	0.424	0.717	-	0.571	0.005
5	0.333	0.558	-	0.445	0.003
Lysozyme (µg/mL)	**1**	**2**	**3**	**Average**	**Standard Deviation**
0	4.737	4.671	4.371	4.593	0.195
100	2.489	2.189	2.523	2.400	0.184
200	2.900	2.916	2.009	2.609	0.519
300	2.881	2.852	2.356	2.696	0.295
400	3.277	2.930	2.964	3.057	0.192
500	3.118	3.217	3.458	3.265	0.174
DMSO (%)	**1**	**2**	**3**	**Average**	**Standard Deviation**
0	2.381	2.443	1.926	2.250	0.282
1	0.931	0.889	0.965	0.928	0.038
2	0.893	0.797	0.778	0.823	0.062
3	0.536	0.344	0.507	0.462	0.103
4	0.537	0.527	0.553	0.539	0.013
5	0.461	0.444	0.426	0.444	0.018

Table C26 (continue): Results of ELISA for screening experiments

LPS (µg/mL)	1	2	3	Average	Standard Deviation
0	3.544	3.754	3.333	3.544	0.298
5	3.083	2.686	2.876	2.882	0.199
10	5.102	4.835	4.504	4.814	0.300
15	4.478	4.293	4.662	4.478	0.185
20	4.012	3.886	3.450	3.783	0.295
25	3.420	2.272	4.382	3.358	1.057

Table C27: Results of ELISA for pre-screening experiments (DMSO)

DMSO (%)	1	2	Average	Standard Deviation
0	0.443	0.465	0.454	0.015
0.5	0.443	0.561	0.502	0.084
1.0	0.906	0.839	0.873	0.048
1.5	1.085	1.265	1.175	0.128
2.0	1.192	1.451	1.322	0.183
2.5	1.750	1.805	1.777	0.039

Table C28: Experimental results of central composite design (Run 1)

Experiment	1		2		3	
Time (h)	Viable cell density (cells/mL)	MAb titer (µg/mL)	Viable cell density (cells/mL)	MAb titer (µg/mL)	Viable cell density (cells/mL)	MAb titer (µg/mL)
0	210,000	0.174	202,500	0.141	185,625	0.205
24	552,500	2.786	557,500	2.408	540,000	2.466
48	1,200,000	6.629	1,496,875	6.316	1,359,375	5.599
72	1,465,625	10.038	1,417,500	11.153	1,570,625	10.131
Experiment	**4**		**5**		**6**	
Time (h)	Viable cell density (cells/mL)	MAb titer (µg/mL)	Viable cell density (cells/mL)	MAb titer (µg/mL)	Viable cell density (cells/mL)	MAb titer (µg/mL)
0	255,000	0.163	209,063	0.218	229,688	0.209
24	571,250	2.210	602,500	2.837	640,000	2.462
48	1,400,000	5.678	1,403,125	4.117	1,518,750	4.153
72	1,474,375	10.334	1,330,000	7.547	1,544,375	8.387
Experiment	**7**		**Control**			
Time (h)	Viable cell density (cells/mL)	MAb titer (µg/mL)	Viable cell density (cells/mL)	MAb titer (µg/mL)		
0	196,875	0.221	193,125	0.127		
24	572,500	3.106	518,750	2.625		
48	1,346,875	5.676	1,015,625	3.684		
72	1,745,625	13.011	1,688,750	7.483		

Table C29: Experimental results of central composite design (Run 2)

Experiment	1		2		3	
Time (h)	Viable cell density (cells/mL)	MAb titer (µg/mL)	Viable cell density (cells/mL)	MAb titer (µg/mL)	Viable cell density (cells/mL)	MAb titer (µg/mL)
0	149,063	0.109	174,375	0.096	161,250	0.103
24	428,750	1.118	292,500	0.775	433,750	0.925
48	700,000	3.150	400,000	2.153	832,500	2.252
72	1,642,500	5.182	768,750	4.080	1,923,750	4.154
Experiment	4		5		6	
Time (h)	Viable cell density (cells/mL)	MAb titer (µg/mL)	Viable cell density (cells/mL)	MAb titer (µg/mL)	Viable cell density (cells/mL)	MAb titer (µg/mL)
0	153,750	0.112	187,500	0.093	207,188	0.115
24	398,750	1.127	433,750	0.767	397,500	0.924
48	610,000	2.721	902,500	2.899	547,500	2.105
72	1,301,250	5.079	1,702,500	4.423	1,370,000	4.405
Experiment	7		Control			
Time (h)	Viable cell density (cells/mL)	MAb titer (µg/mL)	Viable cell density (cells/mL)	MAb titer (µg/mL)		
0	188,438	0.116	165,000	0.074		
24	390,000	0.892	437,500	0.858		
48	840,000	2.572	930,000	2.463		
72	1,606,250	4.684	1,916,250	4.440		

Table C30: Experimental design layout and the data of responses for the optimization

Experiment	LPS concentration (μg/mL)	MAb titer (μg/mL)	Average specific MAb production rate (pg/cell.h)
1	12	7.61	0.1470
2	8	7.62	0.1647
3	9	7.14	0.1229
4	12	7.71	0.1472
5	11	5.99	0.1052
6	10	6.40	0.1107
7	8	8.85	0.1566

The data are averages of two independent runs.

Price of LSD medium supplemented with LPS

Price of 1 mg of LPS (from *E. coli*, 026:B6) obtained from Sigma online catalog on September 2010 = RM 190

Concentration of LPS required in 1 L of medium = 8 μg/mL

Thus, the mass of LPS required in 1 L of medium

$$= 8\frac{\mu g}{mL} \times 1L \times \frac{1000mL}{1L} = 8000\mu g = 8mg$$

And, a total of 8 bottles of LPS are required

$\therefore$ The price of medium after addition of LPS = 8 × RM 190 + RM 39.03 = **RM 1559.03**

The increment of price = $\frac{RM1559.03}{RM39.03} = 40\text{-fold}$

Table C31: Experimental results of the verification run

Experiment	1		2	
Time (h)	Viable cell density (cells/mL)	MAb titer (µg/mL)	Viable cell density (cells/mL)	MAb titer (µg/mL)
0	175,313	0.083	177,188	0.087
24	336,250	1.056	318,750	1.158
48	330,000	3.698	330,000	3.010
72	670,000	5.936	635,000	6.427
Experiment	**Control 1**		**Control 2**	
Time (h)	**Viable cell density (cells/mL)**	**MAb titer (µg/mL)**	**Viable cell density (cells/mL)**	**MAb titer (µg/mL)**
0	165,000	0.100	159,375	0.098
24	405,000	1.341	476,250	1.857
48	827,500	4.635	825,000	3.463
72	1,826,250	7.897	1,194,375	6.711

Table C32: Maximum MAb titer and the average specific MAb production rate of the verification run

Type of culture	Responses	1	2	Average	Standard Deviation
With LPS	**Maximum MAb titer (µg/mL)**	5.936	6.427	6.181	0.348
	Average specific MAb production rate (pg/cell.h)	0.2414	0.2650	0.2532	0.0167
Control	**Maximum MAb titer (µg/mL)**	7.897	6.711	7.304	0.839
	Average specific MAb production rate (pg/cell.h)	0.1627	0.1527	0.1577	0.0071

Raw data for operational parameters screening and optimization

Table C33: Spinner flask culture of hybridoma 192 in LSD (250 mL flask, 40 rpm)

Run 1				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	226,875	249,375	91.0	0.132
23	247,500	310,000	79.8	0.225
29	255,000	312,500	81.6	0.356
47	403,125	541,875	74.4	0.464
53	545,625	650,625	83.9	0.554
71	1,046,875	1,206,250	86.8	0.906
77	1,296,875	1,496,875	86.6	1.025
96	761,250	1,745,625	43.6	3.666
Run 2				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	229,688	251,250	91.4	0.140
23	220,000	313,750	70.1	0.155
29	255,000	317,500	80.3	0.298
47	459,375	598,125	76.8	0.429
53	543,750	684,375	79.5	0.585
71	1,243,750	1,437,500	86.5	0.961
77	1,406,250	1,581,250	88.9	1.125
96	520,625	1,688,750	30.8	3.538

Table C34: Spinner flask culture of hybridoma 192 in LSD (100 mL flask, 40 rpm)

Run 1				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	248,438	261,563	95.0	0.330
23	525,000	548,750	95.7	0.799
29	711,250	732,500	97.1	0.853
47	1,400,000	1,434,375	97.6	0.968
50	1,637,500	1,678,125	97.6	1.025
54	1,815,000	1,841,250	98.6	1.077
71	1,225,000	1,855,000	66.0	2.144
77	705,000	1,730,000	40.8	3.016
96	75,000	1,880,000	4.0	4.458
Run 2				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	242,813	255,938	94.9	0.245
23	523,750	547,500	95.7	0.662
29	711,250	745,000	95.5	0.701
47	1,359,375	1,412,500	96.2	1.035
50	1,506,250	1,540,625	97.8	1.060
54	1,755,000	1,822,500	96.3	1.144
71	1,315,000	1,910,000	68.8	3.873
77	720,000	1,845,000	39.0	4.189
96	0	1,930,000	0.0	4.426

Table C34 (continue): Spinner flask culture of hybridoma 192 in LSD (100 mL flask, 40 rpm)

Run 3				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	219,375	240,938	91.1	0.549
22	297,500	340,000	87.5	0.893
29	385,000	412,500	93.3	0.950
46	827,500	882,500	93.8	1.774
50	965,000	1,030,000	93.7	1.916
54	1,152,500	1,210,000	95.2	2.230
70	1,698,750	1,815,000	93.6	2.819
73	1,601,250	1,822,500	87.9	3.001
78	1,578,750	1,841,250	85.7	3.072
94	307,500	1,653,750	18.6	6.791
Run 4				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	216,563	235,313	92.0	0.624
22	287,500	313,750	91.6	0.789
29	345,000	377,500	91.4	1.036
46	735,000	775,000	94.8	1.508
50	942,500	990,000	95.2	1.629
54	1,172,500	1,215,000	96.5	1.686
70	1,792,500	1,893,750	94.7	3.366
73	1,672,500	1,796,250	93.1	3.409
78	1,537,500	1,743,750	88.2	3.650
94	558,750	1,747,500	32.0	5.778

Table C35: Spinner flask culture of hybridoma 192 in LSD (100 mL flask, 75 rpm)

Run 1				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	261,563	270,938	96.5	0.145
24	705,000	725,000	97.2	0.251
27	783,750	800,625	97.9	0.266
30	907,500	931,875	97.4	0.316
48	2,006,250	2,031,250	98.8	0.385
51	2,317,500	2,358,750	98.3	0.424
54	2,505,000	2,570,000	97.5	0.449
72	478,125	2,356,875	20.3	1.958
Run 2				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	226,875	233,438	97.2	0.158
24	705,000	725,000	97.2	0.280
27	750,000	765,000	98.0	0.299
30	851,250	868,125	98.1	0.360
48	2,000,000	2,025,000	98.8	0.543
51	2,118,750	2,167,500	97.8	0.573
54	2,280,000	2,360,000	96.6	0.689
72	641,250	2,469,375	26.0	2.823

Table C35 (continue): Spinner flask culture of hybridoma 192 in LSD (100 mL flask, 75 rpm)

Run 3				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	219,375	232,500	94.4	0.669
18	206,250	235,000	87.8	0.673
24	206,250	233,750	88.2	0.785
42	455,625	506,250	90.0	1.252
45	596,250	682,500	87.4	1.337
49	673,125	721,875	93.2	1.587
66	1,468,750	1,562,500	94.0	2.025
69	1,466,250	1,560,000	94.0	2.096
73	1,413,750	1,545,000	91.5	2.238
90	620,000	1,700,000	36.5	3.780
Run 4				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	182,813	193,125	94.7	0.625
18	206,250	237,500	86.8	0.806
24	227,500	266,250	85.4	0.917
42	472,500	519,375	91.0	1.216
45	583,125	618,750	94.2	1.383
49	628,125	695,625	90.3	1.520
66	1,496,875	1,568,750	95.4	3.064
69	1,507,500	1,560,000	96.6	3.287
73	1,586,250	1,698,750	93.4	3.488
90	695,000	1,750,000	39.7	5.542

Table C36: Maximum MAb titer and the average specific MAb production rate of hybridoma 192 in spinner culture with LSD

Type of culture flask and stirring speed	Responses	1	2	3	4	Average	Standard Deviation
250 mL	**μ**	0.033	0.034	-	-	0.034	0.001
40 rpm	**r**	0.0617	0.0576	-	-	0.0597	0.0029
100 mL	**μ**	0.037	0.039	0.037	0.040	0.038	0.002
40 rpm	**r**	0.0994	0.1115	0.1919	0.1714	0.1436	0.0451
100 mL	**μ**	0.043	0.044	0.045	0.047	0.045	0.002
75 rpm	**r**	0.0355	0.0524	0.1304	0.1793	0.0994	0.0674

Key : μ = Specific growth rate (1/h); r = Average specific MAb production rate (pg/cell.h)

Surface-to-volume ratio of spinner flasks

$$\frac{Surface\ \ area}{Volume} = \pi(radius)^2 / \pi(radius)^2.(height\ of\ solution)$$

$$= \frac{1}{height\ \ of\ \ solution}$$

For 250 mL spinner flask, diameter = 7.75 cm;

height of solution at 90 mL = 1.9 cm

$$\therefore \frac{Surface\ \ area}{Volume} = \frac{1}{1.9} = 0.526 cm^{-1}$$

height of solution at 230 mL = 4.9 cm

$$\therefore \frac{Surface\ \ area}{Volume} = \frac{1}{4.9} = 0.204 cm^{-1}$$

height of solution at 250 mL = 5.2 cm

$$\therefore \frac{Surface\ \ area}{Volume} = \frac{1}{5.2} = 0.192 cm^{-1}$$

For 100 mL spinner flask, diameter = 6.30 cm;

height of solution at 90 mL = 2.9 cm

$$\therefore \frac{Surface\ \ area}{Volume} = \frac{1}{2.9} = 0.345 cm^{-1}$$

Table C37: Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 1				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	165,938	179,063	92.7	0.080
19	341,250	367,500	92.9	0.127
23	452,500	480,000	94.3	0.140
27	608,750	638,750	95.3	0.155
43	752,500	872,500	86.2	0.255
47	657,500	780,000	84.3	0.261
51	612,500	760,000	80.6	0.278
67	387,500	757,500	51.2	0.418
74	292,500	777,500	37.6	0.362
91	87,500	777,500	11.3	0.424
Experiment 2				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	143,438	149,063	96.2	0.461
22	153,750	272,500	56.4	0.675
25	112,500	211,250	53.3	0.710
41	180,000	318,750	56.5	0.837
44	195,000	315,000	61.9	0.891
65	123,750	326,250	37.9	1.511
69	92,500	286,250	32.3	1.593
89	66,250	311,250	21.3	2.430

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 3				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	245,625	255,000	96.3	0.232
4	249,375	254,063	98.2	0.225
20	326,250	342,500	95.3	0.243
23	415,000	430,000	96.5	0.220
27	443,750	466,250	95.2	0.254
44	512,500	680,000	75.4	0.508
50	459,375	748,125	61.4	0.542
68	69,375	635,625	10.9	0.738
Experiment 4				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	225,000	244,688	92.0	0.156
3	282,188	313,125	90.1	0.161
19	276,250	316,250	87.4	0.239
23	301,250	347,500	86.7	0.269
27	317,500	357,500	88.8	0.342
43	425,000	548,750	77.4	0.367
46	275,625	429,375	64.2	0.387
49	175,000	325,000	53.8	0.595

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 5				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	192,188	212,813	90.3	0.144
18	306,250	326,250	93.9	0.153
22	418,750	442,500	94.6	0.156
26	488,750	520,000	94.0	0.169
43	676,875	723,750	93.5	0.291
46	742,500	813,750	91.2	0.373
49	806,250	864,375	93.3	0.512
67	917,500	1,045,000	87.8	0.828
70	912,500	1,090,000	83.7	0.895
91	525,000	865,625	60.6	1.244
96	431,250	843,750	51.1	1.342
114	177,500	842,500	21.1	2.343

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 6				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	226,875	235,313	96.4	0.397
20	342,500	370,000	92.6	0.376
25	401,250	422,500	95.0	0.398
43	675,000	748,125	90.2	0.762
46	770,625	836,250	92.2	0.795
49	807,500	885,000	91.2	0.848
67	1,222,500	1,392,500	87.8	1.690
70	1,226,667	1,370,000	89.5	1.775
74	1,153,333	1,368,333	84.3	2.319
91	412,500	1,662,500	24.8	10.013
Experiment 7				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	134,063	138,750	96.6	0.311
17	251,250	266,250	94.4	0.333
24	310,000	322,500	96.1	0.362
41	423,750	453,750	93.4	0.623
45	504,375	530,625	95.1	0.740
49	570,000	596,250	95.6	0.771
65	900,000	960,000	93.8	1.257
68	965,000	1,032,500	93.5	1.300
73	1,112,500	1,215,000	91.6	1.400
89	418,750	1,234,375	33.9	7.822

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 8				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	164,063	180,000	91.1	0.804
18	182,500	355,000	51.4	0.955
24	132,500	310,000	42.7	1.023
40	160,000	383,750	41.7	1.045
47	180,000	392,500	45.9	1.108
65	377,500	647,500	58.3	1.167
68	437,500	665,000	65.8	1.299
72	495,000	712,500	69.5	1.664
90	756,250	1,100,000	68.8	1.719
93	987,500	1,221,875	80.8	2.668
96	1,040,625	1,318,750	78.9	3.252
113	1,143,750	1,473,750	77.6	5.910
117	1,275,000	1,522,500	83.7	8.204
120	1,398,750	1,687,500	82.9	12.831
137	660,000	2,028,750	32.5	15.534

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 9				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	177,188	180,000	98.4	0.171
21	217,500	316,250	68.8	0.187
29	177,500	333,750	53.2	0.201
46	198,750	418,750	47.5	0.206
52	201,250	411,250	48.9	0.215
70	551,250	830,625	66.4	0.479
74	622,500	849,375	73.3	0.496
77	661,875	885,000	74.8	0.464
94	1,262,500	1,522,500	82.9	1.147
97	1,597,500	1,796,250	88.9	1.182
101	1,983,750	2,227,500	89.1	1.239
118	1,680,000	2,454,375	68.4	3.259
144	235,000	2,340,000	10.0	3.760
Experiment 10				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	250,313	259,688	96.4	0.804
18	217,500	492,500	44.2	0.955
25	196,250	455,000	43.1	1.023
43	236,250	587,500	40.2	1.045
47	253,750	481,250	52.7	1.108
51	303,750	543,750	55.9	1.167
68	622,500	984,375	63.2	1.299
71	757,500	1,035,000	73.2	1.664
73	811,875	1,048,125	77.5	1.719
91	1,368,750	1,734,375	78.9	2.668
94	1,440,625	1,790,625	80.5	3.252
115	1,544,375	2,270,625	68.0	5.910
122	713,125	1,894,375	37.6	8.204

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 11				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	211,875	227,813	93.0	0.249
19	301,250	340,000	88.6	0.303
23	322,500	343,750	93.8	0.314
27	346,250	363,750	95.2	0.342
43	582,500	620,000	94.0	0.617
47	600,000	647,500	92.7	0.706
51	680,000	735,000	92.5	0.819
67	487,500	926,250	52.6	2.500
75	165,000	1,147,500	14.4	5.505
Experiment 12				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	182,813	193,125	94.7	0.220
17	240,000	258,750	92.8	0.269
21	303,750	323,750	93.8	0.330
25	361,250	382,500	94.4	0.352
41	430,000	472,500	91.0	0.615
45	522,500	570,000	91.7	0.772
49	631,250	709,375	89.0	1.264
65	577,500	836,250	69.1	4.210
73	281,250	877,500	32.1	6.946

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 13				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	135,938	142,500	95.4	0.461
21	307,500	327,500	93.9	0.544
28	395,000	416,250	94.9	0.582
45	579,375	624,375	92.8	0.596
49	646,875	671,250	96.4	0.662
53	778,125	810,000	96.1	0.673
69	1,100,000	1,171,875	93.9	2.097
73	1,159,375	1,278,125	90.7	2.420
77	1,265,625	1,421,875	89.0	3.767
93	735,000	2,095,625	35.1	5.364
Experiment 14				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	202,500	212,813	95.2	0.087
22	498,750	518,750	96.1	0.119
26	675,000	698,750	96.6	0.151
30	796,250	818,750	97.3	0.179
46.5	1,210,000	1,260,000	96.0	0.234
50	1,287,500	1,337,500	96.3	0.240
70	2,152,500	2,310,000	93.2	0.451
74	2,295,000	2,433,750	94.3	0.479
95	2,595,000	3,170,000	81.9	0.563
99	2,356,875	3,459,375	68.1	0.640

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 15				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	221,250	241,875	91.5	0.568
21	517,500	661,250	78.3	0.599
29	517,500	667,500	77.5	0.664
45	968,750	1,118,750	86.6	1.172
49	1,168,750	1,315,625	88.8	1.215
53	1,320,000	1,458,750	90.5	1.272
70	1,680,000	1,859,375	90.4	2.249
73	1,771,875	1,951,250	90.8	2.476
93	515,000	2,275,000	22.6	5.108
Experiment 16				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	167,813	177,188	94.7	0.278
18	328,750	345,000	95.3	0.435
22	358,750	385,000	93.2	0.489
25	413,750	437,500	94.6	0.508
42	712,500	742,500	96.0	0.594
45	860,000	907,500	94.8	0.798
66	780,000	1,451,250	53.7	2.309
68	675,000	1,653,750	40.8	2.874
90	80,000	1,720,000	4.7	5.617

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 17				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	195,000	206,250	94.5	0.084
17	280,000	390,000	71.8	0.160
24	276,250	415,000	66.6	0.187
41	408,750	545,625	74.9	0.203
44	461,250	615,000	75.0	0.213
48	510,000	650,625	78.4	0.219
65	693,750	915,625	75.8	0.351
68	746,875	950,000	78.6	0.426
89	1,146,250	1,461,250	78.4	0.756
92	1,111,250	1,456,875	76.3	0.788
113	290,000	1,450,000	20.0	7.620
Experiment 18				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	148,125	158,438	93.5	0.176
18	333,750	345,000	96.7	0.206
21	390,000	407,500	95.7	0.213
25	447,500	460,000	97.3	0.224
42	817,500	872,500	93.7	0.550
45	940,000	972,500	96.7	0.567
48	1,037,500	1,077,500	96.3	0.613
66	690,000	1,672,500	41.3	6.922
68	626,250	1,923,750	32.6	10.556

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 19				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	185,625	194,063	95.7	0.203
23	213,750	307,500	69.5	0.330
26	265,000	357,500	74.1	0.333
29	332,500	403,750	82.4	0.345
47	532,500	670,000	79.5	0.658
50	590,000	712,500	82.8	0.712
70	885,000	1,031,250	85.8	1.367
74	956,250	1,087,500	87.9	1.422
94	715,000	1,570,000	45.5	7.220
98	540,000	1,890,000	28.6	9.162
Experiment 20				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	211,875	218,438	97.0	0.188
20	452,500	471,250	96.0	0.206
24	522,500	532,500	98.1	0.215
28	636,250	655,000	97.1	0.231
44	1,040,625	1,090,625	95.4	0.431
48	1,225,000	1,259,375	97.3	0.459
52	1,462,500	1,496,875	97.7	0.540
68	1,001,875	1,675,625	59.8	5.685
76	498,750	2,051,875	24.3	11.637

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 21				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	225,938	235,313	96.0	0.163
21	437,500	466,250	93.8	0.235
25	515,000	527,500	97.6	0.253
29	565,000	582,500	97.0	0.277
45	993,750	1,040,625	95.5	0.654
49	1,359,375	1,403,125	96.9	0.737
53	1,487,500	1,553,125	95.8	1.449
69	651,875	1,745,625	37.3	6.533
74	336,875	1,955,625	17.2	14.022
Experiment 22				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	160,313	167,813	95.5	0.124
18	332,500	352,500	94.3	0.147
22	383,750	393,750	97.5	0.151
26	417,500	442,500	94.4	0.156
42	717,500	760,000	94.4	0.382
46	815,000	840,000	97.0	0.390
50	870,000	935,000	93.0	0.416
66	1,601,250	1,661,250	96.4	1.091
70	1,627,500	1,743,750	93.3	1.122
74	1,537,500	1,871,250	82.2	1.613
90	320,000	2,115,000	15.1	4.260

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 23				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	239,063	247,500	96.6	0.114
21	446,250	470,000	94.9	0.166
25	517,500	533,750	97.0	0.177
29	620,000	643,750	96.3	0.165
45	1,012,500	1,034,375	97.9	0.450
49	1,234,375	1,253,125	98.5	0.502
53	1,406,250	1,450,000	97.0	0.530
69	1,347,500	1,833,125	73.5	2.817
74	761,250	2,065,000	36.9	3.615
Experiment 24				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	171,563	183,750	93.4	0.149
21	358,750	385,000	93.2	0.163
25	400,000	435,000	92.0	0.169
29	513,750	541,250	94.9	0.181
46	846,875	871,875	97.1	0.459
49	987,500	1,021,875	96.6	0.502
52	1,150,000	1,203,125	95.6	0.551
70	1,907,500	2,108,750	90.5	2.151
73	1,898,750	2,200,625	86.3	2.444
94	348,750	2,036,250	17.1	9.472

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 25				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	161,250	162,188	99.4	0.144
17	323,750	327,500	98.9	0.163
23	371,250	380,000	97.7	0.208
29	435,000	441,250	98.6	0.256
45	735,000	750,000	98.0	0.294
48	802,500	830,000	96.7	0.489
53	870,000	912,500	95.3	0.593
69	1,350,000	1,410,000	95.7	0.730
72	1,391,250	1,451,250	95.9	0.888
93	1,200,000	1,785,000	67.2	2.175
96	1,085,000	1,915,000	56.7	3.199
117	286,875	1,940,625	14.8	3.692
Experiment 26				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	176,250	184,688	95.4	0.158
20	330,000	347,500	95.0	0.206
24	387,500	400,000	96.9	0.226
28	436,250	458,750	95.1	0.236
45	700,000	740,000	94.6	0.606
48	775,000	807,500	96.0	0.650
51	840,000	887,500	94.6	0.709
69	716,250	1,413,750	50.7	5.216
72	322,500	1,511,250	21.3	9.937

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 27				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	148,125	154,688	95.8	0.319
22	271,250	292,500	92.7	0.423
26	292,500	313,750	93.2	0.428
42	395,000	422,500	93.5	1.106
45	465,000	520,000	89.4	1.173
48	557,500	597,500	93.3	1.270
66	675,000	806,250	83.7	4.534
72	705,000	922,500	76.4	5.355
90	157,500	1,041,250	15.1	14.057
Experiment 29				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	184,688	197,813	93.4	0.311
19	282,500	306,250	92.2	0.319
24	305,000	331,250	92.1	0.353
27	317,500	332,500	95.5	0.356
44	525,000	580,000	90.5	0.846
47	537,500	592,500	90.7	0.969
50	577,500	632,500	91.3	1.402
68	781,250	959,375	81.4	3.731
71	756,250	990,625	76.3	4.404
91	170,000	1,435,000	11.8	7.780

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 28				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	143,438	156,563	91.6	0.268
18	297,500	322,500	92.2	0.328
22	326,250	341,250	95.6	0.360
43	640,000	667,500	95.9	0.914
46	712,500	747,500	95.3	1.007
49	777,500	802,500	96.9	1.067
66	1,496,250	1,548,750	96.6	1.862
70	1,567,500	1,601,250	97.9	2.009
74	1,683,750	1,736,250	97.0	2.276
90	1,635,000	2,065,000	79.2	5.075
96	1,220,000	2,200,000	55.5	6.867
114	438,750	2,216,250	19.8	8.697

Table C37 (continue): Bioreactor culture of hybridoma 192 in LSD for operational parameters screening and optimization

Experiment 30				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	178,125	182,813	97.4	0.246
21	403,750	426,250	94.7	0.263
25	428,750	443,750	96.6	0.310
29	457,500	470,000	97.3	0.331
45	1,030,000	1,060,000	97.2	0.915
50	1,182,500	1,212,500	97.5	0.963
53	1,247,500	1,270,000	98.2	1.024
69	1,295,000	1,855,000	69.8	6.709
72	896,875	2,126,250	42.2	7.093
94	70,000	2,120,000	3.3	13.242
Experiment 31				
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**
0	200,625	213,750	93.9	0.266
18	336,250	362,500	92.8	0.499
23	416,250	437,500	95.1	0.751
27	500,000	532,500	93.9	0.842
43	935,000	970,000	96.4	0.976
46	1,110,000	1,145,000	96.9	1.030
49	1,290,000	1,330,000	97.0	1.202
67	1,824,375	1,868,125	97.7	1.389
70	2,060,625	2,205,000	93.5	2.622
89	680,625	2,289,375	29.7	14.287

Table C38: Experimental design layout and the data of responses for screening and optimization of bioreactor operational parameters

Run	Aera. rate (vvm)	Stirr. speed (rpm)	pH	Temp (°C)	DO (%)	Nv (cells/mL)	μ (1/h)	p (μg/mL)	r (pg/cell.h)
1	0.10	70.00	6.80	37.00	70.00	800000	0.0440	2.495	0.0569
2	0.10	70.00	7.40	33.00	70.00	200000	0.0291	2.430	0.1619
3	0.02	100.00	6.80	33.00	30.00	530000	0.0238	0.738	0.0208
4	0.10	70.00	6.80	33.00	30.00	430000	0.0180	0.595	0.0285
5	0.10	100.00	6.80	33.00	70.00	917500	0.0301	2.343	0.0334
6	0.10	100.00	7.40	33.00	30.00	1226670	0.0256	10.013	0.1482
7	0.02	100.00	7.40	33.00	70.00	1150000	0.0290	7.822	0.1515
8	0.02	100.00	6.80	37.00	70.00	1400000	0.0290	9.352	0.1133
9	0.02	70.00	6.80	37.00	30.00	2000000	0.0470	3.760	0.0339
10	0.06	85.00	7.10	35.00	50.00	1700000	0.0400	8.204	0.0842
11	0.10	100.00	7.40	37.00	70.00	700000	0.0270	5.505	0.1588
12	0.02	70.00	7.40	37.00	70.00	631250	0.0235	6.946	0.2200
13	0.06	85.00	7.10	35.00	50.00	1300000	0.0330	5.364	0.0787
14	0.02	70.00	6.80	33.00	70.00	2700000	0.0360	0.520	0.0032
15	0.06	85.00	7.10	35.00	50.00	1750000	0.0400	5.108	0.0516
16	0.02	100.00	7.40	37.00	30.00	1100000	0.0360	5.617	0.1134
17	0.02	70.00	7.40	33.00	30.00	1150000	0.0230	7.620	0.1093
18	0.10	70.00	7.40	37.00	30.00	1100000	0.0420	10.556	0.2484
19	0.10	100.00	6.80	37.00	30.00	1000000	0.0240	9.162	0.1632
20	0.10	85.00	7.10	35.00	50.00	1600000	0.0370	11.494	0.1807
21	0.06	85.00	7.10	35.00	30.00	1505000	0.0345	14.022	0.2483
22	0.06	85.00	7.10	35.00	50.00	1630000	0.0353	4.260	0.0566
23	0.06	85.00	7.10	35.00	70.00	1520000	0.0327	3.615	0.0555
24	0.06	70.00	7.10	35.00	50.00	1910000	0.0353	9.472	0.1084
25	0.06	85.00	7.10	33.00	50.00	1450000	0.0279	3.692	0.0381
26	0.06	85.00	7.10	37.00	50.00	1050000	0.0310	9.937	0.2470
27	0.02	85.00	7.10	35.00	50.00	710000	0.0258	14.057	0.3594
28	0.06	85.00	6.80	35.00	50.00	1750000	0.0345	8.697	0.0815
29	0.06	85.00	7.40	35.00	50.00	781250	0.0221	7.780	0.1770
30	0.06	85.00	7.10	35.00	50.00	1500000	0.0371	13.242	0.1936
31	0.06	100.00	7.10	35.00	50.00	2100000	0.0345	14.287	0.1569

Key

☐	-	Screening experiment
▭	-	Optimization experiment
Aera. rate	-	Aeration rate
Stir. speed	-	Stirring speed
Temp	-	Temperature
DO	-	Dissolved oxygen
Nv	-	Maximum viable cell density
μ	-	Specific growth rate
p	-	Maximum MAb titer
r	-	Average specific MAb production rate

Table C39: Verification bioreactor cultures of hybridoma 192 in LSD

Run 3								
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (μg/mL)**	**GLU conc. (mM)**	**LAC conc. (mM)**	**GLN conc. (mM)**	**AMM conc. (mM)**
0	180,000	186,563	96.5	0.455	19.85	2.59	6.19	1.62
20	362,500	381,250	95.1	0.678	17.25	7.13	5.88	2.36
24	426,250	441,250	96.6	0.802	16.50	9.11	5.48	2.86
42	702,500	732,500	95.9	1.642	10.04	17.85	3.44	3.20
45	830,000	845,000	98.2	1.892	8.21	22.35	3.16	3.56
48	1,037,500	1,057,500	98.1	2.694	6.30	24.75	2.84	4.65
64	780,000	1,462,500	53.3	13.785	0.66	32.80	1.18	5.65
67	483,750	1,522,500	31.8	17.020	0.12	35.90	1.06	6.02

Table C39 (continue): Verification bioreactor cultures of hybridoma 192 in LSD

Run 1								
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**	**GLU conc. (mM)**	**LAC conc. (mM)**	**GLN conc. (mM)**	**AMM conc. (mM)**
0	177,188	183,750	96.4	0.294	20.85	3.12	5.75	3.72
19	320,000	333,750	95.9	0.416	18.00	5.89	5.70	4.06
23	390,000	410,000	95.1	0.506	16.95	7.17	5.30	4.13
27	432,500	446,250	96.9	0.589	14.95	7.94	4.75	4.41
43	702,500	727,500	96.6	0.895	11.20	15.30	3.68	5.06
48	925,000	952,500	97.1	1.052	9.38	20.85	3.28	5.43
51	987,500	1,052,500	93.8	1.189	7.20	22.95	2.72	5.80
67	1,282,500	1,698,750	75.5	5.094	0.78	34.30	1.17	6.06
72	423,750	1,533,750	27.6	7.446	0.11	35.60	0.89	6.17
Run 2								
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**	**GLU conc. (mM)**	**LAC conc. (mM)**	**GLN conc. (mM)**	**AMM conc. (mM)**
0	189,375	208,125	91.0	0.606	18.60	3.82	6.33	2.96
16	312,500	337,500	92.6	1.001	16.95	7.43	5.53	3.13
24	511,250	537,500	95.1	1.119	12.75	9.91	4.34	3.23
40	1,027,500	1,080,000	95.1	1.833	7.47	22.25	3.43	3.89
44	1,184,375	1,228,125	96.4	2.155	4.98	28.70	3.25	4.56
48	1,334,375	1,393,750	95.7	2.914	2.29	32.75	2.65	5.61
64	306,250	1,548,750	19.8	11.356	0.21	32.70	1.40	6.57

Table C40: Responses of the verification bioreactor cultures

Run	**1**	**2**	**3**	**Average**	**Standard Deviation**
Specific growth rate (1/h)	0.0334	0.0410	0.0346	0.0363	0.0041
Maximum viable cell density (cells/mL)	1.50×10^6	1.41×10^6	1.10×10^6	1.34×10^6	0.21×10^6
Maximum MAb titer (µg/mL)	7.446	11.356	17.02	11.941	4.814
Average MAb production rate (pg/cell.h)	0.1513	0.2498	0.4179	0.2730	0.1348

Raw data for scale up to pilot scale bioreactor

Calculations involved in scaling up to 20 L

For 2 L bioreactor, D_i (impeller diameter) = 5.5 cm, D_T (tank diameter) = 11.9 cm, H_m (medium height) = 11.3 cm and H_i (impeller height from the vessel bottom) = 4.7 cm,

$$\therefore \quad \frac{D_i}{D_T} = \frac{5.5}{11.9} = 0.462$$

$$\frac{H_m}{D_T} = \frac{11.3}{11.9} = 0.950$$

$$\frac{H_i}{H_m} = \frac{4.7}{11.3} = 0.416$$

$$\frac{D_T}{H_i} = \frac{11.9}{4.7} = 2.532$$

For 20 L bioreactor, D_i = 15 cm, D_T = 26.55 cm,

$$\therefore \quad \frac{D_i}{D_T} = \frac{15}{26.55} = 0.565$$

$$\left(\frac{H_m}{D_T}\right)_{2L} = 0.950 = \left(\frac{H_m}{D_T}\right)_{20L}$$

$$H_m = 0.950 \times 26.55 = 25.22cm$$

$$\left(\frac{D_T}{H_i}\right)_{2L} = 2.532 = \left(\frac{D_T}{H_i}\right)_{20L}$$

$$H_i = \frac{26.55}{2.532} = 10.49cm$$

$$\left(\frac{H_i}{H_m}\right)_{20L} = \frac{10.49}{25.22} = 0.416 = \left(\frac{H_i}{H_m}\right)_{2L}$$

The stirring speed used in 2 L bioreactor, N_2 = 100 rpm and $(D_i)_2$ = 5.5 cm = 0.055 m, while the stirring speed used in 20 L bioreactor = N_{20} and $(D_i)_{20}$ = 15 cm = 0.15 m,

∴ Impeller tip speed in 20 L bioreactor = impeller tip speed in 2 L bioreactor

$$\pi N_{20}(D_i)_{20} = \pi N_2(D_i)_2$$

$$N_{20} = \frac{N_2(D_i)_2}{(D_i)_{20}} = \frac{100\ rev/\min \times \frac{1\min}{60\sec} \times 0.055}{0.15} = 0.611\frac{rev}{\sec} \times 60\sec/\min = 36.7\frac{rev}{\min} \approx 37\,rpm$$

Table C41: 20 L bioreactor cultures of hybridoma 192 in LSD

Run 1								
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**	**GLU conc. (mM)**	**LAC conc. (mM)**	**GLN conc. (mM)**	**AMM conc. (mM)**
0	171,563	177,188	96.8	0.437	24.70	5.00	6.61	3.08
20	411,250	428,750	95.9	0.583	19.55	10.05	5.57	3.22
23	480,000	502,500	95.5	0.634	18.45	11.25	5.26	3.37
40	759,375	778,125	97.6	0.834	12.80	23.00	4.57	3.70
44	912,500	937,500	97.3	1.059	10.30	26.25	4.06	4.11
47	1,050,000	1,065,625	98.5	1.223	8.69	30.05	3.76	4.48
64	1,623,125	1,806,875	89.8	3.570	1.18	43.60	1.48	4.96
70	726,250	2,135,000	34.0	9.251	0.18	43.20	1.10	5.56
Run 2								
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**	**GLU conc. (mM)**	**LAC conc. (mM)**	**GLN conc. (mM)**	**AMM conc. (mM)**
0	245,625	262,500	93.6	0.652	20.150	5.72	6.10	3.11
21	502,500	525,000	95.7	1.186	16.550	14.25	5.30	3.18
25	646,875	675,000	95.8	1.249	14.650	15.95	5.06	3.77
29	759,375	806,250	94.2	1.461	13.500	20.45	5.00	3.57
45	1,534,375	1,581,250	97.0	2.293	3.150	40.20	3.32	3.74
50	1,980,000	2,066,250	95.8	2.861	0.300	42.40	2.44	4.23
54	1,601,250	2,047,500	78.2	4.993	0.180	41.80	1.98	4.75
70	236,250	2,017,500	11.7	7.675	0.150	39.95	1.56	5.24

Table C41 (continue): 20 L bioreactor cultures of hybridoma 192 in LSD

Run 3								
Time (h)	**Viable cell density (cells/mL)**	**Total cell density (cells/mL)**	**% Viability**	**MAb titer (µg/mL)**	**GLU conc. (mM)**	**LAC conc. (mM)**	**GLN conc. (mM)**	**AMM conc. (mM)**
0	185,625	195,938	94.7	0.599	21.20	4.90	6.23	2.60
20	451,250	463,750	97.3	0.838	17.15	10.60	5.63	2.77
24	523,750	532,500	98.4	1.056	15.95	12.85	5.36	2.78
27	572,500	596,250	96.0	1.149	15.40	15.15	5.01	3.23
44	1,071,875	1,100,000	97.4	1.915	6.96	29.10	3.81	3.27
48	1,268,750	1,300,000	97.6	2.147	4.21	33.95	3.33	4.32
51	1,590,625	1,628,125	97.7	2.228	2.18	36.95	2.85	4.47
68	485,625	1,898,750	25.6	12.420	0.16	40.85	1.54	4.70

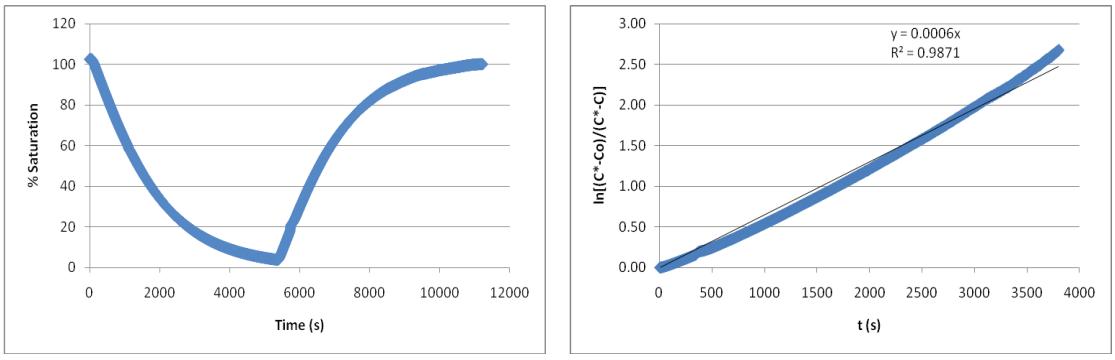

Figure C2: Results of k_La study in 2 L bioreactor (Run 1)

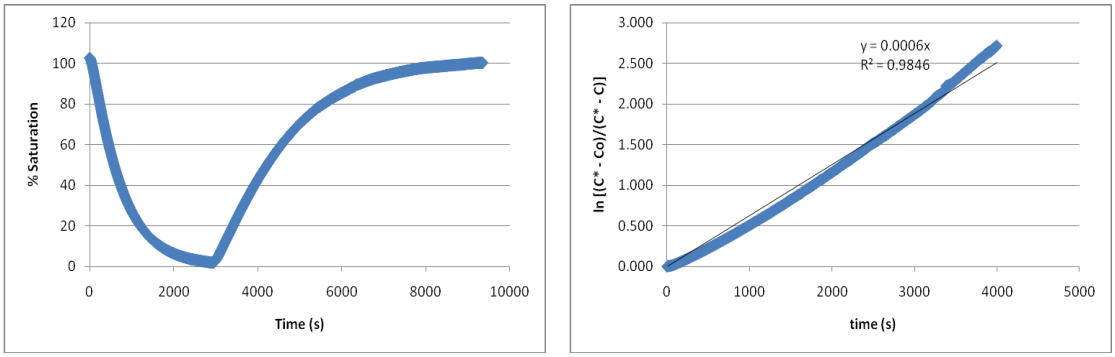

Figure C3: Results of k_La study in 2 L bioreactor (Run 2)

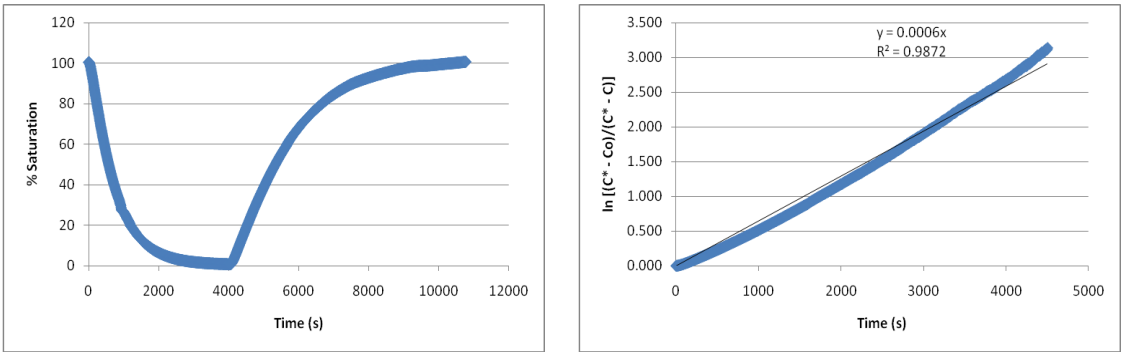

Figure C4: Results of k_La study in 2 L bioreactor (Run 3)

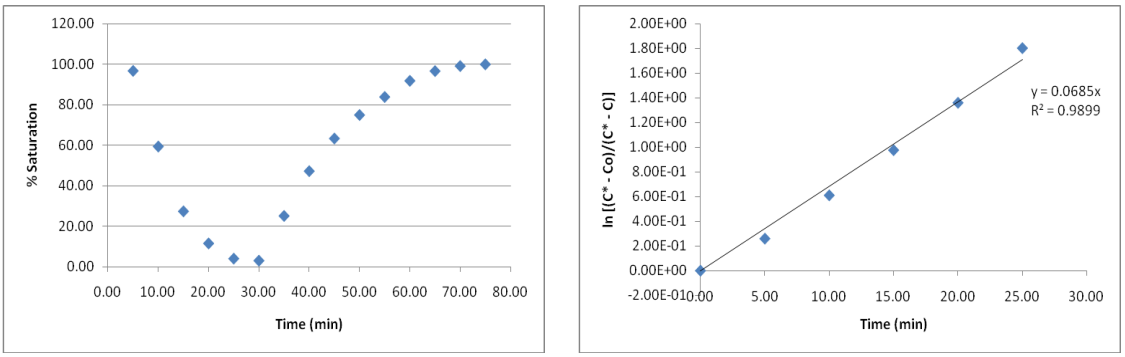

Figure C5: Results of k_La study in 20 L bioreactor (Run 1)

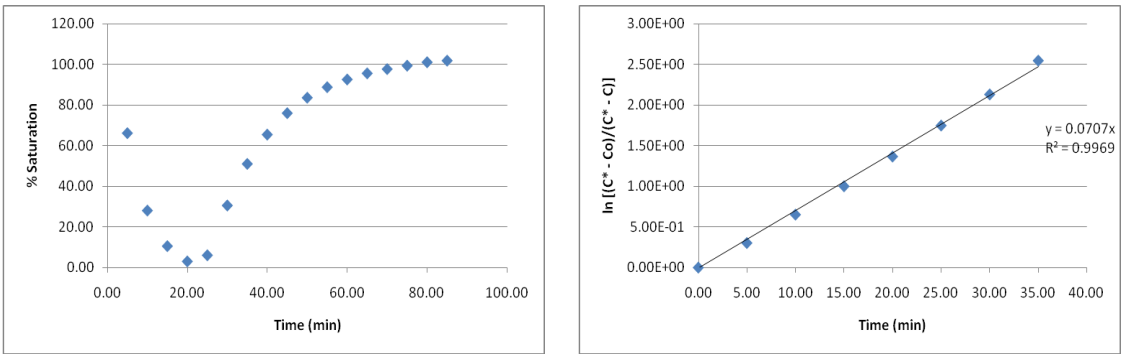

Figure C6: Results of k_La study in 20 L bioreactor (Run 2)

Table C42: Comparison of k_La values between 2 L and 20 L bioreactors

Size of bioreactor	k_La (s^{-1})				
	1	2	3	Average	Standard Deviation
2 L	0.0006	0.0006	0.0006	0.0006	0.0000
20 L	0.0011	0.0012	-	0.0012	0.0000

Table C43: Optical density of purified MAb (after Protein G column) from 20 L bioreactor at 280 nm

Fraction collected	Optical Density
1	0.014
2	0.013
3	0.051
4	0.083
5	0.035
6	0.017
7	0.014
8	-0.005
9	0.005
10	0.005
11	0.006
12	0.007

Table C44: Optical density of purified MAb (after Protein G column) from 20 L bioreactor versus dilution factor using ELISA coated with antigen

Dilution Factor	Optical density
0.125	1.1160
0.0625	0.8672
0.03125	0.6207
0.015625	0.4460
0.007813	0.3415
0.003906	0.1507
0.001953	0.1002
0.000977	0.0019

Table C45: Optical density of purified MAb (after Protein G column) from 20 L bioreactor in specificity test

17-OHP concentration (ng/mL)	Optical Density
4000	0.0025
3000	0.0477
2000	0.1534
1500	0.2162
1000	0.2930
750	0.3337
500	0.3402
375	0.3537
250	0.3697