Robust Strategy Development for Primary Recovery of High Cell Density Mammalian Cell Cultures Using Ultra Scale-Down Models

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I, Daria Popova, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

For:

Natalia Lvovna Voyteleva,

Maria Konstantinovna Voyteleva

And my Mother

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Abstract

Advances in mammalian cell culture have led to significant increases in cell concentrations and product titres. This has placed considerable demands on primary recovery and purification operations through associated increases in solids and impurity loads. These pose an additional challenge for the development of efficient and cost-effective processing strategies for the future. The work presented in this thesis focuses on the development of a methodology for the identification and selection of future-proof primary recovery technologies for the harvest of high cell concentration mammalian cell cultures.

A survey questionnaire was developed to define industry needs in terms of desired primary recovery operational criteria as well as changes in cell culture material expected over the next decade. The industrial responses were used to form the base assumptions for the cell culture conditions used in the experimental work. The cell concentration and product titre expected in 2020 ranged up to $100x10^6$ cells/mL and 20 g/L respectively. Impurity removal targets for the future primary recovery technologies were also quantified, where HCP removal was found to be of highest priority, followed by DNA and then aggregate removal.

An integral element of the research focused on establishing a methodology to mimic future feed profiles to primary recovery stages. This resulted in the creation of a set of cell culture test materials (CCTM) where key variables including cell concentration, cell viability, product and host cell protein (HCP) load could be manipulated individually. The methodology involved using tangential flow filtration (TFF) to generate a range of target cell concentrations, induced cell apoptosis to achieve the target viabilities and ultrafiltration of purified harvests to create a range of Immunoglobulin G_1 (IgG_1) and HCP concentrations. The CCTM methodology was used at the 70L scale to quantify the reproducibility of the material, which was found to be within 10% of the target cell and HCP concentrations and 15-20% of the target cell culture viability.

The CCTM methodology was then integrated with scale-down experimentation, multi-attribute decision-making, process economics and facility fit considerations to provide a systematic framework for the screening of primary recovery technologies with the potential for future use. These included three current (centrifugation and depth filtration combinations including 05SP, 10P and 30ZA) and two alternative technology options (charged TFF hollow fibre modules including Bio-Optimal MF-SL and QSD). HCP removal levels of approximately 10% were reached across all the tested technologies, however removal of specific HCP groups varied. Up to 99% DNA removal was achieved using the QSD, with lower levels of DNA removal using the other options.

MADM analysis as well as a selection based on current technology performance criteria showed that only two options met the yield and purity criteria: Bio-Optimal MF-SL and the QSD. The centrifugation and the 30ZA option met the purity criteria but not the yield criteria. Further economic evaluation of the options showed the centrifugation and 30ZA option to be the least cost-effective across the 2,000L, 10,000L and 20,000L scale scenarios and to not fit the facility constraints, which were set based on a typical existing large-scale facility. The Bio-Optimal MF-SL option was the most cost-effective option across the 2,000-20,000L scales of operation.

Economic and MADM analyses of the alternative technologies were used to identify primary recovery options for the future. The QSD was found to provide greater capability for DNA removal prior to purification operations, however remained a costly option. The Bio-Optimal MF-SL offered a similar level of solids removal but was more cost-effective. Specific process requirements as well as other technology alternatives have to be taken into account indvidually to further select a cost-effective and appropriate technology choice.

The effects of cell concentration, cell culture viability and HCP concentration on primary recovery performance were then quantified using a Design of Experiments (DoE) approach. Multivariate data analysis wasused to determine predictive correlations capturing the impact of changes in cell culture composition on primary recovery performance. Ultimately this allowed identification of the most cost-effective primary recovery technology options to be centrifugation and depth filtration at cell concentrations below $50x10^6$ cells/mL, the Bio-Optimal MF-SL at cell concentrations in the range of 60-$80x10^6$ cells/mL and titres below 8 g/L, and the QSD at cell concentrations above $80x10^6$ cells/mL and titres above 12 g/L.. The work in this thesis demonstrates a systematic integrated economic and experimental approach to identifying robust primary recovery technologies for future mammalian cell culture processes.

Contents

List of Tables

List of Figures

Abbreviations and Symbols

Abbreviation	Description
ADD	7-Amino-Actynomycin D
ATF	Alternating Tangential Flow
BHK	Baby Hamster Kidney
BSA	Bovine Serum Albumin
CCTM	Cell Culture Test Material
CHO	Chinese Hamster Ovary
COG	Cost Of Goods
CTC	Counter Current Tangential Chromatography
DHFR	DiHydroFolate Reductase
DoE	Design of Experiments
EBA	Expanded Bed Adsorption
FACS	Fluorescence-Activated Cell Sorting
Fc	Constant fragment of mAb
GBM	metastatic Breast Cancer Glioblastoma
GS	Glutamine Synthetase
HAMA	Human Antimouse Antibody Response
HCP	Host Cell Protein
HVAC	Heating Ventilation and Air Conditioning
ICH	International Conference on Harmonisation
I_gG_1	Immunoglobulin G1
I_gG_4	Immunoglobulin G4

IVCC	Integral Viable Cell Concentration
LE	Large Enterprises
mAb	monoclonal Antibody
MADM	Multi Attribute Decision Making
mCRC	metastatic Colorectal Cancer
mRCC	metastatic Kidney Cancer
MSX	Methylamine Sulphoximine
MTX	Methotrexate
NADPH	Nicotinamide adenine dinucleotide phosphate
NS0	NonSecreting murine myeloma
NSCLC	non-small cell lung cancer
PBS	Phosphate Buffered Saline
PEI	Polyethylenimine
PER.C6	Human cell culture cell line
QbD	Quality by Design
QTPP	Quality Target Product Profile
QSD	Qyu Speed D
RDF	Rotating Disc Filter
RMU	Relative Monetary Units
rpm	Revolutions per minute
SME	Small and Medium-sized Enterprises
SP2/0	*Spodoptera frugiperda*
STR	Stirred Tank BioReactor
TCD	Total Cell concentration (also known as “cell density)
TFF	Tangential Flow Filtration

TMP	Trans Membrane Pressure
USD	Ultra Scale-Down
WFI	Water for Injection
WHO	World Health Organisation

Symbol	**Description**	**Units**
C_{Ra}	Equivalent numerical value of the confidence rating basis	%
D	Cell concentration	cells mL^{-1}
d_{min}	diameter	m
f_a	Frequency of option a selected in an answer to a question	
F_{OD}	Sample post primary recovery, optical density	Absorption units
FR	Confidence weighted frequency rating	%
g	Acceleration due to gravity	ms^{-2}
i	Option in available in a given question (survey)	
n	Number of different available answers	
N	Number of discs in a disc stack centrifuge	
V_g	Particle settling velocity	ms^{-1}
Q	Volumetric flowrate	m^3s^{-1}
Q_0	Q at time = 0	m^3/s
Q_t	Q at time = t	m^3/s
r_i	Inner disc radius	m
r_o	Outer disc radius	m

R_m	Clean membrane resistance	m^{-2}
R_p	Resistance of growing deposit	m^{-2}
R_{p0}	Resistance of the initial deposit	m^{-2}
t	Time	s
V	Total filtrate volume	m^3
V_{max}	Volume filtered before blockage	m^3
S	Solids removal	%
TP	Total protein concentration	gL^{-1}
TY	Total product concentration	gL^{-1}
PFR	Confidence weighted frequency rating	%
V_s	Starting concentration volume	m^3
V_c	Final concentration volume	m^3
SP	As subscript, spiked	
P_A	Actual figure for a performance attribute	
P_{min}	Minimum figure for the same performance attribute	
P_{MAX}	Maximum value for the same performance attribute	
R	As subscript, desired	
S	As subscript, starting	
P_a	Performance for attribute A	
OR	Overall Rank	
w_i	Weighted value for a performance attribute	
Ni	Normalised value for a performance attribute	
V_T	Cell culture volume	L

M_{WC}	Wet mass per cell	g
S_R	Solids fraction remaining	%
S_H	Solids holding capacity	kg
U	Number of centrifuge units	
Q_{Cin}	Maximum feed flowrate to a large scale centriguge	L/h
m_{Pin}	Product mass	kg
V_{max}	Maximum throughput through a depth filter	L/m^2
S_F	Safety Factor	
A_M	Membrane area per filter module	
N_{MSK}	Number of filter modules per skid	
V_{DFhup}	Hold up volume	L
Y_{CP}	Unit specific product concentration yield	g/L
OP_A	Minimum space required for operation	m
M_{LW}	Mass of liquor in the sediment	kg
N_P	Number of discharge operations	
V_W	Total liquor loss in the sediment	L
t_{PCent}	Processing time	H
V_{DFin}	Volume in (depth filtration)	L
A_{DF}	Total membrane area required (depth filter)	m^2
N_m	Number of modules required	
Y_{DF}	Product Yield (depth filtration)	g/L
A_{FSP}	Floor space required	m^2
A_{TFF}	Total membrane area required (TFF filter)	
V_{TFFp}	Permeate volume (TFF)	L

Y_{TFF}	Product Yield (TFF filtration)

Greek Symbols	**Description**	**Units**
α	Half the canonical angle of the disc in a centrifuge	^{o}
β	Membrane specific constant	
Δρ	Density difference	kgm^{-3}
μ	Dynamic viscosity	$kgms^{-1}$
Σ	Equivalent clarification area in the centrifuge	m^2
ω	Angular velocity	$rads^{-1}$

Chapter 1: Introduction

The high demand for cost-effective therapeutic protein products has been a continuing driver for process improvements, especially in the case of monoclonal antibody production. Due to the biological similarity and the complexity of these products, platform processes based largely on mammalian cell culture have been designed and implemented for the majority of the products over the last 20 years. Advances in cell culture methods and technologies have resulted in significant increases in cell culture titres and densities. The current workhorses of primary recovery operations can struggle to perform effectively with these high cell concentration cultures. This challenge potentially calls for a new strategy to cope with increased biomass, product and impurity levels from cell culture. This thesis describes the development of a methodology to screen rapidly and evaluate primary recovery technologies for their capacity for robust and cost-effective operation in the context of high cell concentration cultures. This introductory chapter provides an overview of the historical developments in the biopharmaceutical industry, an analysis of the current and future challenges in primary recovery of mammalian cell culture broth, as well as an overview of key primary recovery unit operations and cost modelling considerations. Section 1.2 provides an overview of the past and current trends in recombinant protein production. Sections 1.3 and 1.4 discuss key trends in mammalian cell culture and primary recovery methods and Section 1.5 discusses the main considerations which apply to recombinant protein production. Bioprocess cost modelling considerations are discussed in Section 1.6. Finally, the aims and organisation of the thesis are presented.

1.1 Trends in recombinant protein production

This section will explore the recent trends in recombinant protein production and some of the reasons behind them. The section will begin with a discussion of the recent production trends in terms of market demand, as well as more specific manufacturing trends, such as the recent issues experienced in the contract manufacturing industry. This will be followed by an overview of the steps involved in a typical mammalian cell culture process as well as some of the drivers behind the volume requirements and other trends observed in processing over the years. The section will conclude with an overview of the regulatory requirements in the biotechnology industry and their implications for processing.

1.1.1 Trends in recombinant protein production

The age of recombinant proteins began with the arrival of insulin between 1982 and the early 1990's, which soon became the fastest growing branch of the biopharmaceutical market (Johnson, 2003; Jenkins, 2007). By 2000, investment in biotech was at the highest point ever observed before this poing, resulting in a large number of products entering clinical trials (Huggett *et al.,* 2009). However, shortly following the market successes, the

demand for Enbrel (Fc-fusion protein, produced by Immunex) exceeded the manufacturing capacity to the extent that the drug had to be rationed, leading to the acquisition of Immunex by Amgen. (Mallik, Pinkus, and Sheffer, 2006). The fear of such potential underproduction had caused many biopharmaceutical companies to start mammalian cell construction projects, and was believed to have led to an increasing trend towards outsourcing production (Ransohoff, 2007). Today it is very common for biopharmaceutical companies to source capacity from contract manufacturing organisations (CMOs) so as to mitigate risks and either to delay the significant capital outlays required to build a manufacturing facility or to supplement existing in-house facilities (Kelley, 2009). Therefore, considerations and assumptions made throughout these studies will come from the stand point of a CMO as opposed to a bioprocess developer, when considering strategies for technology changes and facility scenarios.

Today, monoclonal antibodies (mAbs) are the most prevalent class of recombinant protein (Kelley, 2009). The majority of mAb products target chronic diseases where the biggest sector is oncology (Pavlou and Belsey, 2005), requiring long term treatment (see Table 1.1). An intermittent high dose is typically required (e.g., every 4-6 weeks), which over the course of a year can result in a demand of 1-20 g of mAb per patient. This is considered to be a high long term dose requirement. The next section provides an overview of the processing trends in mAb production which will allow the industry to meet these dose requirements and demands in the past.

Table 1.1: *An overview of the top 4 current mAbs, their target dose, administration method and predicted performance in the near future (Bloomsberg consensus forecast, 2011, sales of mAb from primary companies: Janssen (J&J), Roche and Abbott).*

Predicted Position	mAb (commercial name)	Company	Type	Targets	Maximum dose (g/person/ year)	Administration method	Predicted sales in 2015 ($ m)
1	Humira	Abbott	TNF-inhibitor	Rheumatoid Arthritis Juvinile Ideopathic Arthritis Psoriatic Arthritis Ankylosing Spondilitis Crohn's disease Plaque Psoriasis	2 1	Pen, Prefilled Syringe	9,875
2	Avastin	Roche	VEGF-A	Metastatic Colorectal Cancer (mCRC) Non–Small Cell Lung Cancer (NSCLC) Metastatic breast cancer Glioblastoma (GBM) Metastatic Kidney Cancer(mRCC)	11 22	Intravenous	8,046
3	Herceptin	Roche	anti HER2	Adjuvant treatment for breast cancer Metastatic treatment for breast cancer Metastatic treatment gastric cancer	9	Intravenous	6,352
4	Remicade	Janssen (J&J)	TNFα inhibitor	Crohn’s Disease Ankylosing Spondylitis Psoriatic Arthritis Plaque Psoriasis Ulcerative Colitis Rheumatoid Arthritis	3 1	Intravenous	3,009

1.1.2 Trends in mAb production

The first mouse mAb to reach clinical practice in 1986 (OKT-3), presented significant challenges for manufacturing and commercialisation as many of the early clinical trial patients experienced immune reactions due to the generation of their own anti-mouse antibodies (Human Antimouse Antibody response-HAMA). The emergence of mAbs as a major, established biopharmaceutical class was due to the development of humanisation technology combined with efficient and cost-effective mammalian cell culture production and recovery methods (Ezzell, 2001).

Following the success of the first antibodies in the market, there was a need to identify a strategy for the quick transition of the emerging molecules into the clinic. Several of the larger biotech companies, including Amgen (who had just acquired Immunex), designed and implemented a platform approach to mAb process development (Gombotz and Shire, 2010). A platform approach requires definition of a set of robust unit operations and methodologies to yield consistent results when used for the production of different products or cell lines. The typical platform seen today in mammalian cell monoclonal antibody production includes fed-batch cell culture, followed by primary recovery, protein A chromatography, polishing chromatography steps, viral filtration and ultrafiltration before progressing further to formulation (see Figure 1.1) (Kelley, 2009). Cell culture operations typically fall into upstream operations (in some cases primary recovery operations are included as well), primary recovery and purification operations are typically considered part of downstream operations. These are designed to purify the product to meet the regulatory requirements for human administration. However, 2007 survey results showed that the greatest concern across CMOs was “the physical capacity of downstream equipment” (Langer, 2009). This implied that the contract manufacturing industry was experiencing strain on the performance of downstream operations as well as pressure to increase capacity. This pressure on purification can be critical as the failure to meet regulatory requirements automatically precludes a product from the market. Although downstream unit operations are typically designed to carry out a specific role (e.g. cell removal, DNA removal etc.) the process as a whole must also be designed to meet the relevant regulatory requirements. These will be discussed in the following section, which provides a summary of the key regulatory aspects directly related to the processing trends discussed in this chapter.

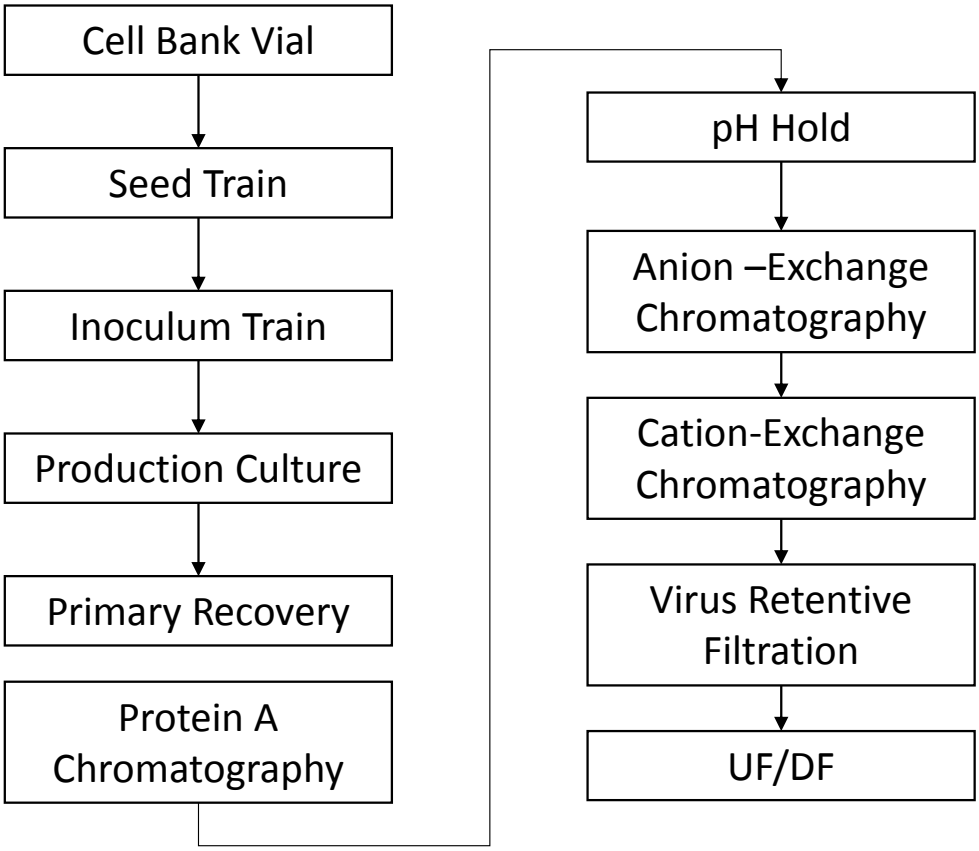

Figure 1.1: *Conventional mAb process flow sheet for bulk drug substance. Typically titres reached during fermentation are in the range of 2-5 g/L. Typical yields after the protein A capture step yields 70-80% (Adapted from Kelley et al., 2009).*

1.1.3 Requirements in the Biotechnology Industry

Recombinant protein production processes are designed not only to fulfil the market demand of the particular product in terms of product yield and yearly volume requirement, but equally to fulfil requirements regarding product quality.

Primary requirements for pharmaceutical protein products mainly focus on minimal requirement levels for host cell proteins, product variants (aggregates, glycosylation variants), DNA, viruses, endotoxins, leachables and small molecules added during the fermentation/cell culture and the purification processes. Host cell protein (HCP) acceptance levels are within micrograms per gram of product (Jin *et al.*, 2010). If levels of these impurities in feed streams to processes increase, higher removal capacity will be required by the downstream operations to match set criteria levels. Thus, any inconsistency or increase in HCP production upstream may require a change of technology or increase in downstream operational performance. Alternatively, host cell engineering or more appropriate cell line selection can be implemented as a long term strategy.

Product aggregates cause varying levels of immunogenic response and therefore are also considered an impurity. The allowed content of product aggregates depend on the product, however on average the final product should contain <5% of protein product variants (these may include deaminated, oxidised and incorrectly glycosylated forms). The acceptable levels depend on the bioactivity shown during clinical trials. The DNA levels set by the World Health Organisation (WHO) are at ≤10 µg per dose. In terms of virus particles, regulation at the moment states that the viral levels should be less than 1 virus particle per million doses. Viral removal is typically achieved by nanofiltration and low pH hold steps. Endotoxin levels are usually set to less than 5EU/kg of patient weight, whilst leachable levels are set typically at ≤1-10µg/mL (Rosenberg, 2006). The current reported improved analytical methods are able to provide an increasing amount of detail in terms of impurity characterisation as well as allowing a greater sensitivity in detection (Jin *et al.*, 2010; Wang *et al.*, 2010.). This can potentially put greater pressure on downstream operations in setting higher specification targets for impurity removal (HCP removal in particular), subsequently requiring a greater level of impurity clearance from the unit operations used. Increases in impurity levels have been associated with increases in cell culture titre and cell concentration. In order to discuss the role of primary recovery operations in meeting regulatory requirements in mAb production processes, cell culture and primary recovery operations will be discussed in detail in the following sections, beginning with an introduction to mammalian cell culture expression systems.

1.2 Trends in mammalian cell culture

Prior to discussing primary recovery operations this section will explore the key properties of the typical types of feed to primary recovery operations. For the purpose of this research it is important to review the trends in titre increase as well as the methodologies used to achieve them.

Mammalian cells are predominantly used for recombinant protein production due to their effective protein folding mechanisms and consistent ability to perform complex post translational modifications (Jenkins, 2007). Consistency in glycosylation pattern is analogous to product consistency and is essential for regulatory process approval. Those biopharmaceutical proteins that undergo simple modification can be produced using yeast or bacteria, e.g. albumin (Recombumin by Novozymes) and insulin (Lispro by Elli Lilly and Novo Nordisk) (Roach and Woodworth, 2002). However, molecules with more complex glycosylation requirements are more commonly produced using mammalian cell lines. There is a range of mammallian cell lines approved for expression of recombinant proteins, some derived from mouse myeloma (NS0 or SP2/0), baby hamster kidney cells (BHK), Chinese hamster ovary cells (CHO), and a humanised cell line PER.C.6. However 70% of all recombinant protein production is carried out in CHO cell lines which includes all currently

approved monoclonal antibody products (Jayapal, 2007). The reason for this likely stems from early development of recombinant technology being based on expression in heavy and light chains of CHO cell antibodies. Platforms for transfection and selection of high producer cell lines using CHO cells waere well developed which allowed mAb production to take advantage of already established technology used for other recombinant products such as erythropoietin, Factor VIII, plasminogen activator. At the time these recombinant products typically were produced at low expression levels, approximately <1 g/L (Birch, 2005). Consequently, rapid growth in market demand for mAbs and other recombinant proteins (e.g. Fc-fusion proteins) was a key driver to increase scale of the production facilities resulting in large culture volumes of >10,000L (Kelley, 2009).

Reported product titres using CHO cell culture have been increasing over the years, from the 50mg/L region, which the original platform processes were designed for, to 2g/L by mid 90s, reaching greater than 12g/L in 2010 (Bibila and Robinson, 2000; Charaniya *et al.*, 2010). This is thought to be due to the improved understanding of gene expression, growth, metabolism and apoptosis delay (Wurm, 2004).

This section provided examples of significant increases in cell culture titre over the last decade. These increases may have been achieved by increasing specific cell productivity as well as the cell concentration. Both of these mechanisms can impact the load on primary recovery and downstream applications.

In order to explore the range of cell lines likely to require primary recovery processing in the future, the next section will provide a discussion on the expression systems used for mAb production in the past and the key mechanism by which they work.

1.2.1 Mammalian Cell Expression Systems

During the transfer of recombinant DNA, which results in the production of the protein of interest, the efficiency with which the DNA is taken up varies between cell types and selection methods (Kucherlapati and Skoultchi, 1984). Selectable markers are used either to complement host cell deficiencies or to provide resistance to otherwise toxic agents therefore facilitating the detection of the plasmid containing the gene of interest. Several such selection markers have been identified including xanthine, guanine phoshoribosyltransferase, adenosine deaminase, multiple drug resistance (e.g., colchicines, adriamycin, actinomycin), asparagine synthetase, glutamine synthetase (GS) and dihydrofolate reductase mutants (DHFR) (Chapman *et al.*, 1983; Cartier and Stanners, 1990; Choi *et al.*, 1988). Most commonly, commercial cell lines utilise either the DHFR or the GS expression systems.

The DHFR enzyme catalyzes the NADPH-dependent reduction of folate to tetrahydrofolate, which is an essential cofactor in the synthesis of glycine, purines, and thymidine. Enzyme synthesis can be inhibited by the addition of a folate analogue- methotrexate, which leads to a deficiency of thymdylate resulting in cell death (Blakely, 1969). When used as a selection marker, DHFR is introduced into DHFR deficient hosts (which otherwise can only be grown in supplemented media), those which have taken up the plasmid can have resistance to the methotrexate by amplifying the DHFR gene. If the gene of interest is located on the same expression vector it is co-amplified and its presence is detected with the presence of resistance during the addition of methotrexate. One of the advantages of using the DHFR as a selectable marker is the ability to modulate selective pressure by inhibiting gene expression with methotrexate (MTX). The same effect can be achieved also by reducing the enzyme activity which can result in a compensatory increase of expression via an alternative pathway (e.g. copy number increase) (Simonsen and Levinson, 1983).

Codon optimisation has been used in the past as a strategy to increase gene of interest expression, by increasing the translational efficiency of the gene of interest, thus achieving titre increases during the cell line development stage. More recently a different approach emerged which involved “codon deoptimisation” of the DHFR gene in combination with increasing selection pressure by altering the internal mRNA to decrease expression of the DHFR gene (Wernicke and Will, 1992).

GS is another widely used expression system. Glutamine synthetase enzyme converts glutamate and ammonia to glutamine, which is not only an essential amino acid but also is vital for nitrogen detoxification and plays a role in purine biosynthesis. The system works when glutamine is omitted from the media, while maintaining low concentrations methionine sulphoximine (MSX), approximately 50mM, which inhibits indigenous glutamine synthetase favouring the survival of transfected cells. However cells which have acquired the additional copies of glutamine synthetase (also expressing the gene of interest) will survive (Schlokat, 1997).

The GS system was made commercially available by Lonza Biologics in the late 1980s, for use with CHO and NS0 cell lines. By the end of the 90s research showed that the GS system in NS0 cell lines was yielding higher titres than CHO cell lines when optimised (up to 510mg/L in batch culture while in CHO only 270 mg/L) (Birch *et al.*, 1993; Bebbington 1991). The following section will provide an overview of the current trends in titre increases and highlight the systems and cell lines used to achieve the current high titre processes, noting however that CHO cell lines are still one of the most common cell types used today.

1.2.2 Trends in Titre Increases

Improvements in cell line development, media and process optimisation have enabled continuing titre increases in mammalian cell processing. In the 80's reported product titres were reaching 100 mg/L in batch cultures, which increased to 1 g/L over the next 10 years with the development of fed-batch culture (allowing the culture period to double) (Wurm, 2004). Recently titres of up to 12g/L have been reported (Huang *et al.,* 2010).

Reported titre increases have been achieved by increasing the culture time, the specific productivity of cells, increasing the viable cell concentration and a combination of the two effects. Currently the highest reported titre of 13g/L has been achieved in a Fc fragment producing CHO cell line through media optimisation. Non glycosylation mAb producing CHO cell line through medium optimisation also yielded a high titre of 12 g/L. The achieved titre was stated, but no data was provided. In the same sets of experiments, the highest achieved total cell concentrations ranged between 13-15 $x10^6$ cells/mL with specific productivities in the range of 30-50 pg/cell/day. The high titre process was optimised by implementing a screening method to identify the effect of components in three different basal media and four feed media. The screening method reported in this case was aimed at increasing cell mass, enhancing specific productivity and longevity of the cells, which requires balancing increases in specific productivity with the effects on the viability and density of culture (Huang *et al.*, 2010). This is one of the potential reasons for the choice of a lower specific productivity over the maximum reported back in 2004 of 90pg/cell/day (Wurm, 2004).

An increase in specific productivity can be induced by the addition of sodium butyrate to the media. Sodium butyrate is a short chain fatty acid, histone deacetylation inhibitor, and has been used to increase specific productivity of secreted proteins in mammalian cells, including CHO and hybridoma cells (Sung *et al.*, 2004). However, the addition of sodium butyrate causes a depletion of viable cells and an increase in the number of apoptotic cells (Mimura *et al.*, 2001). The addition of sodium butyrate yields most favourable results when introduced in the late exponential phase as the inhibitory effect on cell proliferation is minimised (Hunt *et al.*, 2002). The molar concentration of sodium butyrate also affects the degree of growth arrest observed. Low concentrations of 0.25- 0.5mM have been reported to have no significant effect on cell viability and growth. However higher concentrations of 1-4mM resulted in slow growth and a decrease in cell viability, overall resulting in a maximum of a two-fold decrease in cell concentration at high sodium butyrate concentrations only yielding a cell concentration of $2x10^6$ cells/mL (Goulart *et al.*, 2010). Overall, moderate concentrations of sodium butyrate have been shown to result in up to three-fold increases in titre (Mimura *et al.*, 2001) which suggests a compromise must be struck between the impact its addition has on specific productivity increases, cell concentration and viability.

The following section will discuss the cell concentrations which have been observed over the years and highlight the typical cell concentrations which have been reached to date.

1.2.3 Trends in Cell Concentration Increases

Currently, two culture formats are available for mammalian cells: adherent and suspension. The latter is widely used for industrial recombinant protein production, where three modes of operation can be applied: batch, fed-batch and perfusion. CHO cell culture is predominantly carried out using fed-batch fermentation. Although perfusion culture has been promising in terms of high titre and cell concentration production, product dilution still remains a downside of this mode of operation. Controlled rate perfusion methods have been shown capable of reaching and maintaining cell concentrations of >150x10^6cells/mL over the course of 30-60 days, however this cell concentration has only been achieved using the PER.C.6 cell line (Schirmer *et al.*, 2010; Clincke *et al*., 2013a; Clincke *et al*., 2013b; Karst *et al.,* 2016). Cell cultures carried out using fed batch processes have also been reported to achieve cell concentrations in the range of 10-40x10^6 cells/mL (Singh *et al.,* 2013). The cell concentrations achieved by these different processes vary along with the definition of "high cell concentration culture". In order to capture the full range advancements, independent of the technology used, cell concentrations over 1x10^7 cells/ mL tend to be referred to as "high cell density" in this thesis (Westoby *et al.*, 2011).

As discussed above, the current range of titres achieved during cell culture ranges by roughly 100 fold. Primary recovery operations used today were designed during the first platform development movement, when cell concentrations and titres reached maximum of 1x10^6 cells/mL and cell culture titres averaged 1g/L (Wurm, 2004). Increases in cell concentration lead to an increase in solids content, impurities as well as cell debris, presenting challenges to both primary recovery and purification operations.

Although significant increases in cell concentrations have been achieved, there has been little move towards implementing changes in the technologies used in primary recovery. This implies that current primary technologies provide sufficient efficiency for current processing. However if it is assumed that the trend of increasing cell concentrations and titres continues (Immarino *et al,* 2007; Singh *et al,* 2013; Westoby *et al.,* 2011), the pressure to identify alternative primary recovery operations that may present more cost-effective process options in the future will present an opportunity for future process development (Singh *et at.,* 2013). It is this perceived pressure that forms the motivation for the studies reported in this thesis and leads this introduction to provide an overview on primary recovery operations currently used as platform processing options as well as those seen as alternative technology options.

1.3 Primary recovery of mammalian cell culture

The first steps following mammalian cell culture in recombinant protein processing are traditionally designed to remove the insoluble components of the cell culture broth. This means that the main role of primary recovery operations has been to achieve a sufficient level of clarification to prepare the material for progressing to purification. Centrifugation and depth filtration are the most commonly implemented harvest operations, with tangential flow microfiltration options also considered for use in some cases. The following section will review the typical primary recovery operations as well as the potential alternatives in terms of their theoretical principles and their key features.

1.3.1 Centrifugation

Principles

Centrifugation is a preferred method of solid-liquid separation due to the ease of its scalability and economical operation for large volumes (Maybury *et al.*, 2000). Therefore it tends to be used for scales of 2000L and above (Yavorsky *et al.*, 2003). Although multichamber and tubular bowl centrifuge designs are available, the disc-stack centrifuge is the most commonly used as it accommodates for intermittent discharge of solids during operation and therefore is more effective in clarification of large volumes and higher solid feeds. The centrifuge is able to remove a high percentage of cells and cell debris prior to secondary clarification and subsequent product clarification steps. However it is typically not effective at removing small particles in mammalian cell broths (1-6µm) which will cause fouling of the chromatography column. This is also the reason behind the common utilisation of depth filtration post centrifugation (Kempken *et al.*, 1995). Any improvement in centrifuge performance, regarding increased levels of solids removal or reduced levels of cell disruption during processing, will translate to a reduction in the filter area required to protect the chromatography column. Figure 1.2 provides a cross sectional view a typical disc-stack design.

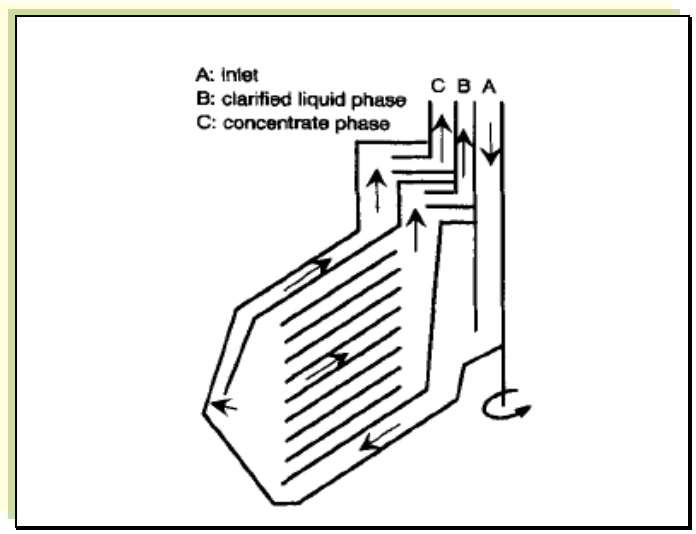

Figure 1.2: Schematic of key flow paths inside the disk stack centrifuge. Liquid feed flows through inlet A, out to the side of the bowl and up between the discs. The solids settle on the disc, making their way back down and up to outlet C, while the clarified liquid flows through to outlet B.

The settling area of a continuous disk stack centrifuge is defined as follows:

$$\Sigma = \frac{2\pi}{3g} \times \omega^2 \times N \times \cot\alpha \times \left(r_o^3 - r_i^3\right) \tag{1.1}$$

Where Σ is the equivalent clarification area in the centrifuge (m^2), g is the acceleration due to gravity (ms^{-2}), ω is the angular velocity ($rads^{-1}$), N is the number of disks, α is half the conical angle of the disk (°), r_o is the outer disk radius (m), r_i is the inner disk radius (m). Sigma theory can be used to predict the performance of the same feed in different centrifuges and at different scales:

$$2V_g = \frac{Q_1}{\Sigma_1} \quad \text{and} \quad \frac{Q_2}{\Sigma_2} \tag{1.2}$$

Where V_g is the particle settling velocity and Q is the volumetric flowrate into the centrifuge (m^3s^{-1}). The minimum diameter of particles which can be separated (d_{mi},, m) can be calculated as stated below:

$$d_{\min} = \sqrt{\frac{Q}{\Sigma} \times \sqrt{\frac{18\mu}{\Delta\rho g}}} \tag{1.3}$$

Where μ is the dynamic viscosity of culture medium ($kgms^{-1}$), Δρ is the density difference between cells and medium (kgm^{-3}) and g is the acceleration due to gravity (ms^{-1}).

Centrifugation features

Through CFD analysis, the feed zones of disk- stack and multichamber centrifuges have been identified as the area resulting in high shear due to the presence of air- liquid interphases. Levels of energy dissipation in a non-hermetic feed zone may reach up to $0.37x10^6 W\ kg^{-1}$ where 99% of the energy has been shown to dissipate to the liquid phase due to the higher density of the liquid in comparison to the air phase (Hoare *et al.*, 1992; Boychyn, 2001). High-energy dissipation results in high shear during operation and subsequent cell breakage and decreased clarification performance. The minimum particle size which can be removed by a centrifuge is a function of the centrifuge bowl design, cell culture properties (viability, density), feed rate and rotational speed (consider equations 1.1, 1.2 &1.3) but is typically >0.5µm.

Shear in the feed zone can be reduced by introducing a hermetic seal, thereby eliminating the air- liquid interphase in the feed zone. This reduces the energy dissipation rates typically to a maximum of $0.019x10^6 W\ kg^{-1}$ and reduces cell breakage, thereby also reducing the number of fine particles present post centrifugation (Hoare *et al.*, 1992; Tait *et al.*, 2009).

Studies using a Westfalia CSA-1 model with hermetic seal, with a working volume of 600 mL showed that the concentration of non-viable cells in the feed broth was linearly correlated with the decrease of clarification efficiency. In addition, centrifugation conditions were shown to have no impact on the quantity of impurities present post centrifugation indicating that shear levels are uniform across the range of centrifugation generating conditions and that the key parameter causing variation is the condition of the feed. Finally it was noted that the % of clarification converged for cell viability levels above 80%. Iammarino *et al.* (2007) suggested to set a minimum viability at >50% for efficient clarification.

Hermetically sealed feed zones are currently available in disk stack and CARR Powerfuge models. The performance of these models has been investigated in terms of levels of shear produced. All models of the CARR Powerfuge showed 69-76% recovery and little shear damage by comparing the DNA concentrations in the centrate to the concentrations in the original cell broth (Lander *et al.*, 2005). Another technique which is easily implemented in most centrifuge designs is the full bowl start up, which reduces the air- liquid interphase, reducing the shear encountered during start up (Hoare *et al.*, 1992).

A study examining the effects of solid content increase in cultures of *Pichia pastoris* on centrifugation performance showed that although some product loss was encountered when increasing the solids content from 2% to 15%, the loss can be offset by the higher dewatering levels achieved from higher solids content feeds. The same study also proposed a method for establishing a window of operation for higher solid content feeds, which can be applied to other host organisms (Salte *et al.*, 2006).

At average cell concentrations relatively low discharge frequencies are typically required, therefore the contribution to product loss is minimal (Maybury *et al.*, 2000). However increasing cell concentrations results in an increase in the solid content of the culture broth which in turn required a greater frequency of desluge, potentially leading to a greater degree of product loss. These inter-relationships emphasise the strong level of interactions that exist between the unit operations engaged in a typical mammalian cell culture based manufacturing process.

1.3.2 Filtration Principles

Pore blockage

Filter capacity is determined by the fouling characteristics of the feed solution, which causes a decay in flowrate during constant pressure operation or an increase in pressure during constant flux operation. Flux decline is said to be caused by one or a mixture of mechanisms including pore blockage, intermediate pore blockage, pore constriction and cake filtration (Hermia, 1982).

Initial flux decline is typically associated with pore blockage or constriction, which is followed by cake filtration. The model developed to reflect this has been demonstrated (Ho and Zydney, 2000), (see equations 1.4-1.6). This model is considered to provide a more accurate description of fouling than the classical filtration models, which are based on just a single pore blocking mechanism (Reis and Zydney, 2007).

$$\frac{Q}{Q_0} = \exp(-\beta t) + \frac{R_m}{R_m + R_p}\left[1 - \exp(-\beta t)\right] \tag{1.4}$$

$$R_m = \frac{1}{L_p} \tag{1.5}$$

$$R_p = \left(R_m + R_{p0}\right)\sqrt{1+\alpha t} - R_m \tag{1.6}$$

Where Q is the volumetric flowrate (m^3s^{-1}) at time t, Q_0 is the initial flowrate (m^3s^{-1}), t is the filtration time (min), R_m is the clean membrane resistance, R_p is the resistance of the growing deposit, R_{p0} resistance of the initial deposit and Lp is the membrane hypraulic permeability.

1.3.3 Depth Filtration

Principles

Depth filtration is designed to achieve cell and cell debris removal through the filter media as opposed to surface removal observed in microfiltration membranes (Yavorsky *et al.*, 2003). It is typically employed in place of, or in combination with, centrifugation in the clarification of mammalian cell culture broths in monoclonal antibody producing processes as well as in the

removal of acidified protein solutions prior to chromatographic processing often employed as prefilters (Singhvi *et al.*, 1996). Depth filtration has also been demonstrated to remove DNA and host cell proteins during clarification of animal cell cultures when using charged depth filtration medium (Yigzaw *et al.*, 2006; Charlton *et al.*, 1999). However where large cell culture volumes are concerned, providing the required membrane area for clarification is economically and operationally inefficient, therefore centrifugation followed by depth filtration tends to be employed during clarification at scales above 2000L (Yavorsky *et al.*, 2003).

Particle removal is achieved by a combination of mechanisms which also contribute to membrane fouling (see Figure 1.3). Cells and cell debris are removed by capture inside pore spaces (pore constriction and plugging), also referred to as depth straining. This occurs when a particle travels down the narrowing pore until it becomes trapped or lodged inside the pore. This applies not only to particles which are the same size as the pore but also to those smaller through a mixture of physical mechanisms (McCabe *et al.*, 1976). This is exploited especially well in graded filter modules, where a mixture of grades is used to form a network of pores that decrease in size through the depth of the filter material. Therefore different sizes of particles are removed at distinct portions within the body of the filter.

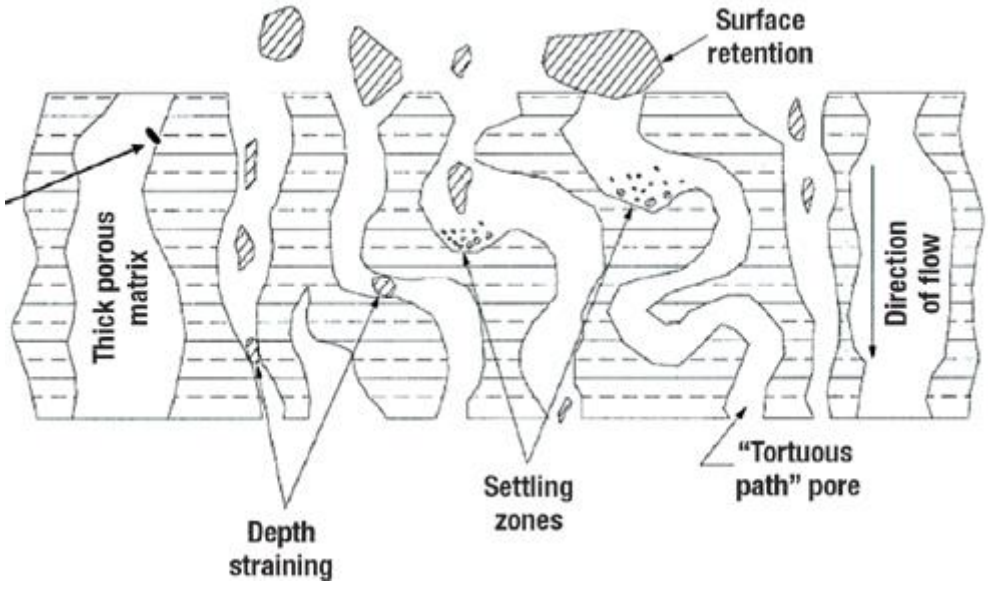

Figure 1.3: Cross section of a depth filter medium demonstrating removal mechanisms (Shukla and Kandula, 2008)

A large portion of the particles present in any given feed are retained at the filter surface. These are typically cells or particles which are larger than the pore size. In turn, other cells or debris which are brought in contact with the pores and particles already attached to the pores, also become attached by van der Waal and other forces. Flow through larger pores is restricted by the presence of smaller particles which collect in the pores, allowing cake to form on top of the membrane, which in turn acts as an additional filter medium. The positive charge often provided by the material effectively binds some DNA, viruses and endotoxins

(Charlton *et al.*, 1999). As a consequence of these multiple mechanisms it is possible for a certain pore size to allow capture of smaller particles than might be expected solely based on the basis of the surface pore rating.

Recovery operations where depth filtration is used generally involve a high-solid containing feed load to the filter where cake filtration by surface retention dominates. A similar situation applies at small scale mammalian cell clarification operations where depth filtration is used as the pre-clarification step. However in larger scale operation (as previously described) where depth filtration is preceded by centrifugation, the feed consists of mainly debris, implying lower solids content and a smaller average particle size.

Filter Media

Typically the filter media used in bioprocessing includes cellulose or polypropylene fibres, where filter aids can be added (e.g., diatomaceous earth, perlite, activate carbon etc). Alternatively, charged filters are manufactured by incorporating charged polymers or ion exchange particles within the material (Reis and Zydney, 2007). Depth filter media does not typically come with an absolute pore size rating unless a membrane layer at the end of the flowpath is included. However the depth filter tends to be followed by an absolute pore size rated filter (typically 0.45µm or 0.2 µm) that ensures the removal of solid particulates (also bacteria in case of the 0.2 µm filter) from the cell culture harvest supernatant.

Modules

Depth filtration units utilised in mammalian cell culture fluid clarification are typically composed of circular replacement cartridges. The lenticular disc arrangement has been common in pre-clarification, where each disc is made from two circular layers of thick filter media bonded together at the outer edge. For example the Zeta Plus™, 3M (previously known as Zeta Plus™, CUNO) depth system first widely advertised in 1997 demonstrates this configuration and are said to provide a 1-2 log removal of DNA (Dorsey, 1997). However these systems were recognised to present some operator, CIP and scaling issues in reviews by competitors and independent review articles. As the discs are stacked vertically hoists may be required in some cases to change the discs. In some cases the filter medium between the discs sagged resulting in contact between the discs.

Product innovation in the depth filtration market has most recently aimed at combating these problems. For example, Millipore had offered the Millistak system to combat the operator problems of vertical stacking configuration. The system comprises of filter graded cassettes stacked in a horizontally placed housing. In the early 2000's the advantages of single use systems had become more widely recognised (Reis and Zydney, 2001), which included a move towards single use depth filtration equipment. Millipore's POD system, similar in configuration to the Millistak was introduced and adopted widely at this time (Lambalot *et al.*,

2006). Similarly the CUNO Zeta Plus™ system evolved to allow single use of the lenticular discs, which were advertised to also achieve HCP removal. The current design of the housing still utilises vertical configuration, which provides greater space efficiency, having improved upon the previous design by incorporating a mechanical system allowing the discs to be loaded in the horizontal position and then mechanically moving the stack into its vertical configuration.

1.4 Alternative Primary Recovery of Mammalian Cell Culture

Optimisation and the development of new, improved primary recovery methods is driven by the possibility for non-protein chromatographic separation (Roush and Lu, 2008). A variety of approaches have been described, roughly dividing into two types of alternatives:

- Those which aid the performance of current operations; and
- Those which offer an alternative operational option.

1.4.1 Tangential Flow Filtration (TFF)

Principles

Tangential flow microfiltration is a competing unit operation to centrifugation and depth filtration in the clarification of mammalian cell culture broths. One of the key advantages it provides is the production of a particle free harvest stream in one unit operation which requires little further clarification prior to application of the clarified material to chromatography as opposed to centrifugation operations which can result in the introduction of additional debris into the stream (Reis and Zydney, 2001).

In tangential flow mode the cells and debris present in the feed flows at a tangent to the filter medium as opposed to directly through it. An applied pressure forces the liquid through the filter medium forming the permeate stream. Particles larger than the pore size remain in the original flow and are swept across the surface of the membrane, forming the retentate. In operation, tangential flow membranes are dominated by concentration polarisation and fouling effects. The filtrate flux through the membrane is limited by the accumulation of a gel layer of cells and debris along the flow path through the membrane. However this can be avoided by exploiting a low flux in the system or taking advantage of inertial lift effects which would help combat the gel effect (Belfort *et al.*, 1994). The consequence of this solution is increased processing times as well as the risks associated with it, including potential effect on product stability, cell viability, impurity release and clarification efficiency.

Membrane media

Tangential flow microfiltration is most commonly accomplished in flat sheet and hollowfibre membranes. Flat sheet membranes are typically cast on non- woven substrate and either have an isotropic or asymmetric structure. Commercially available membrane material includes polysulphone, polyethersulfone, cellulose and hydrophilized polyvinylidene fluoride, which are often modified to provide the required surface properties (e.g., hydrophobicity) (Zeman and Zydney, 1996). Hollowfibre modules are also typically made from polyethersulfone, polysulfone, and polyvinylidien fluoride as well as mixed cellulose esters and polypropylene. Structurally hollow fibers are self-supporting and dense at the lumen, so can be easily cleaned (Reis and Zydney 2007). Polysulphone material tends to be favoured for mammalian cell clarification stages.

Membrane technology can aid fast process development as small scale cassettes are readily available for primary recovery (0.1 m^2 membrane area). However, much like centrifugation the technology is sensitive to culture properties such as cell viability, density and medium components (Westoby *et al.*, 2011). High cell concentrations and low cell viabilities have been suggested to result in high transmembrane pressures. An application of membrane cascades using a series of membrane processes has been suggested to achieve separation of a soluble biologic by exploiting the difference in sieving coefficients at each step resulting in improved separation factors (Lightfoot *et al.*, 2008).

1.4.2 Flocculation

The addition of a flocculation step directly following cell culture is aimed at improving the clarification efficiency during primary clarification, reducing the need for secondary clarification by aggregating the smaller debris. This step has not been widely used in the biotechnology industry as its wide application originates in waste water treatment, however it is expected to bring applied benefits in the future (Shukla and Kandula, 2008). This lack of translation into industrial use may stem from the need to add a processing step that enhances the following filtration stages, however it does not provide a replacement for the filtration operation. Polyelectrolyte induced flocculation has been proposed as an alternative to Protein A chromatography in the separation of a humanised monoclonal antibody from cell culture fluid. This process was shown to achieve similar purity levels to current chromatography based processes and demonstrated the removal of the added agents post product capture using anion exchange chromatography (McDonald *et al.*, 2009).

Flocculation processes have been shown to be highly sensitive to feed stream composition and operating conditions, including pH, ionic strength, depending on the choice of flocculant (Habib *et al.*, 2000). Flocculation is driven by the collisions between the polymer and the

particle, however increased shear rates can cause the flocculated particles to break up into small particles, which have a low re-growth rate after the shear is reduced (Kim, 2001; Yukselen 2004). CHO cell flocs were found to be more shear sensitive in comparison to *E. Coli*, potentially leading to the presence of a large number of small particles within the broth (Han *et al.*, 2003).

Flocculating agents

Cationic polymers tend to be most effective for mammalian cell culture flocculation, to match the overall negative charge of the cells. Therefore the addition of cationic polymers into the cell culture fluid results in electrostatic interactions between the negatively charged cell debris and whole cells in the culture. Examples include polyamines, cationic polysaccharides, chitosan and diallyldimethyl ammonium chloride. The use of polyamines, including polyethyleneimine (PEI) and polyallyamine as cationic polymers to induce the flocculation of host cell impurities as an alternative to column chromatography has been demonstrated. The removal of CHO HCPs has been shown (Ma *et al.*, 2010). PEI was also effectively used for the flocculation of *E. Coli*, (Salt, 1995; Barany and Szepesszentgyörgyi 2004) and yeast cultures (Milburn *et al.*, 1990; Habib *et al.*, 2000). Molecular weight and rate of agitation greatly impact the flocculation efficacy, as opposed to mixing time which has little effect (Habib *et al.*, 2000). The advantages of PEI in industrial application include small volume requirements and low material cost, however the subject of PEI removal from processing streams post clarification is generally avoided in the reviewed publications. It is important to note that PEI removal is required in order to fulfil regulations on human administration in the US and EU.

The use of chitosan as a flocculant in NS0 culture was shown to improve dramatically the clarification achieved by the following centrifugation and increase the capacity of the following depth filter with no adverse effects on the monoclonal antibody recovery or purity. The process was shown to be relatively robust under operation in a pH range of 5-7.5 (Riske *et al.*, 2007). Chitosan has additional benefits in application in biotechnology as it is sourced from non- mammalian in origin and is available in a highly pure form.

1.4.3 Acid Precipitation and Cell Settling

Acid precipitation of cells and cell debris has been explored over the past 20-30 years. Induction of flocculation with the decrease of pH has been shown using centrate derived from hybridoma cell culture. In addition it demonstrated the precipitation of DNA and the combination of clarification with viral inactivation early on in the process (Lydersen *et al.*, 1994). Later it was suggested that it can be carried out directly inside the bioreactor, followed by centrifugation and depth filtration resulting in removal of the precipitate. This was shown to increase capacity of the depth filtration operation. Acid precipitation was also

carried out prior to a centrifugation and a microfiltration step where the flux of the microfiltration operation was shown to be dramatically improved (Roush and Lu, 2008).

Acid precipitation followed by cell settling has been tested by Westoby *et al.*, (2011) for a range of CHO cell lines prior to hollowfibre microfiltration. The combination of low pH with the addition of cobalt chloride showed an increase in product yield, a 3 log removal of DNA, as well as a consistently low TMP at 15 LMH in comparison to non-precipitated culture fluid. HCP reduction was also achieved, however the reduction was insignificant, which was attributed to the hetrogenous nature of the HCP pI as opposed to DNA (Westoby *et al.*, 2011). Although the cell line to cell line variability was not clearly explored, the results showed a promising prospect for the future of cell settling options.

1.4.4 Expanded Bed Absorption (EBA) Chromatography

Expanded bed adsorption has attracted some interest over the years; however it has found little industrial application in recombinant protein recovery operations so far. Drager and Chase first introduced the idea of a fluidised bed in the 1990s, later expanding its use to protein purification (Drager and Chase, 1991). More stable adsorbents were introduced by Amersham Pharmacia Biotech in 1993 named STREAMLINE™, which were later assessed in their ability to clarify mammalian cell broths. Protein A was rated most efficient in all aspects of performance followed by the Chelating matrix which rated highly in CIP performance but exhibited poor cell and debris clarification (Lütkemeyer *et al.*, 2001). However, when human embryonic kidney cell culture was loaded onto a Chelating adsorbent it was shown that cell high cell recovery was achieved in terms of cell number and viability, exhibiting little cell breakage during the process (Poulin *et al.*, 2008). Single use adsorptions chromatography columns have also become available (Lihme *et al.,* 2015), and EBA is still considered in microbial and whey process purification stages (Du *et al.,* 2014; Xu *et al.,* 2014; Hansen *et al.,* 2013)

1.4.5 Counter Current Tangential Chromatography (CTC)

This technology was newly patented in 2009, with an initial demonstration for the separation of bovine serum albumin (BSA) from a protein mixture provided using Macro Prep 25Q anion exchange resin. The resin flows through a series of static mixers and hollowfibre modules. The hollowfibre membranes retain the large resin particles, letting the dissolved species flow through. The solution containing the protein of interest is then released and protein binds to the slurry molecules, while the impurities are removed as waste. Binding, washing and elution buffer flow is counter current to the resin through multiple hollowfibre modules which enables high resolution separation and increase in product yield. Myoglobin impurity removal was shown to be >99% and the product yield to be 94% (Shinkazh *et al.*, 2011). This

technology has potential in cell clarification or perfusion if the design is modified for cell accommodation.

1.4.6 Alternating Tangential Flow Filtration (ATF)

Alternating tangential flow (ATF) filtration has been used in perfusion and concentrated fed-batch modes to achieve high cell concentration cultures of >150x10^6 cells/mL. The processes using a number of cell lines have been shown, including HeLa cells for production of recombinant vaccinia virus (Bleckwenn *et al.,* 2005), PER.C.6 cell line in a process referred to as the XD (Schirmer *et al.,* 2010), and an IgG_1 producing CHO cell line (Clinke *et al.,* 2013). There is currently no publically available data for its implementation specifically as a primary recovery step, however manufacturers have seen to have potential in the area. The following information has been obtained through discussions with the manufacturers of the ATF.

The mechanisms by which the process works ensures low shear processing which is advantageous for processing both high and low viability cultures, however the system is designed for low cross flow rates, which does not cause a problem during cell culture operations.

There are two potential methods of ATF implementation in primary recovery: reduced time harvest and the long harvest. Reduced time harvest involves harvesting over approximately 3-4 hours at high cross flow rates and is suitable for high viability harvests (>50%). However this methodology is not thought to exploit the full potential of the system. In addition it is possible that a secondary clarification stage may be required post ATF.

The long harvest methodology requires a degree of integration of cell culture process and the primary recovery process. The primary recovery operation is thought to commence on the day of harvest, however the process is likely to take more than 20 hours. This methodology is predicted to increase cell culture times as product expression carries on throughout the harvest operation, ensuring that viable cells are intact and producing whilst clarified material is harvested at low cross flowrates. The methodology would be most suited to concentrated batch operations, however it may prove beneficial when implemented within fed-batch cell culture modes of operation.

1.5 Ultra Scale-Down (USD) of Primary Recovery Unit Operations

During early stage bioprocess development, validation, implementing process changes, quality by design principles (QbD), and other work requiring the running of a large number of experiments can result in high investment due to large quantities of material required. Therefore the value of scale down of unit operations has been long recognised and desired (Boychyn, 2001). Although small scale models of a variety of units are available (Maybury *et*

al., 2000), many require a relatively large volume and only allow single experimental runs at a time contributing to cost and time issues related to such studies. Ultra scale-down techniques of unit operations across processing areas have been demonstrated, including cell culture (Korin *et al.*, 2009), clarification (Tait *et al.*, 2009) as well as purification operations (Neal *et al.*, 2003).

1.5.1 USD Centrifugation

Early scale down techniques of centrifugation processes were based on decreasing the volume requirements in a centrifuge by introducing blank/ dummy discs which was shown to reduce the throughput requirements by a factor of 10 (Hoare *et al.*, 1992). However the volume reduction achieved by a direct mimic of centrifugation did not achieve sample requirement reduction to the degree where significant changes to experimental procedure and experimental cost were made. Methods to mimic large scale centrifugation in terms of recovery and performance using a lab scale centrifuge have been described. This method was shown to predict large scale performance well in the case of polyvinyl acetate particles, but not protein precipitates which showed solids breakup during centrifugation (Hoare *et al.*, 1992). However, mimicking centrifugation in a way which incorporates the full functionality of the large scale centrifugation as well as the discontinuous discharge feature has not been carried out to allow small volume utilisation. The smallest currently available model (Westfalia CSA-1) requires a volume of 600 mL, allowing for 250 mL of solids holding (Roush and Lu 2008). Subsequent scale down methods focused on reproducing the shear which the material experiences during the centrifugation operation. Multiple assessments of this scale down techniques have been carried out and improved methods have been introduced. Ultra scale-down of centrifugation has achieved the smallest required sample volume of 20 mL. The principle is based on mimicking shear cell culture is exposed to in a full scale centrifuge and subjecting cell culture to the equivalent shear force by using a rotating disk device (Tait *et al.*, 2009).

Scale up of centrifugation has been carried out using the sigma concept-or the equivalent setting area. A typical scale down model used in a number of scale down studies in described below (Boychyn *et al.*, 2004; Hutchinson *et al.*, 2006; Levy, 1999; Tait *et al.*, 2009). Remaining solids after large scale continuous centrifugation can be determined using equation 1.7.

$$S = \frac{Q_c}{\sum_c} \tag{1.7}$$

This is related to the bench scale test tube centrifugation using equation 1.8:

$$\left(\frac{Q_c}{C_c \sum_c}\right) = \left(\frac{V_B}{t_B C_B \sum_B}\right)$$

(1.8)

Where C_c is an experimentally derived factor 1, C_b is an experimentally derived factor 2, V_B is the volume of material (L), t_B is the centrifugation time (h).

The relationship used to determine the Sigma of a laboratory scale centrifuge was described by Maybury *et al.*, (2000):

$$\sum_B = \frac{V_B \omega (3 - 2x - 2y)}{6g \ln\left(\frac{2r_o}{(r_o + r_i)}\right)} \tag{1.9}$$

Where Σ_B is the equivalent settling area of a bench top centrifuge, V_B is the volume of sample used (mL), ω is the angular velocity ($rads^{-1}$), x is the time required for acceleration (h), y is the time required for deceleration (h), r_i= inner radius (m), r_o= outer radius (m).

r_i and r_o are determined using the following relationships:

$$r_i = \sqrt{(p + (q - L)\cos\vartheta + a\sin\vartheta)^2 + (b)^2} \tag{1.10}$$

(1.11)

$$r_o = \sqrt{(p + q\cos\vartheta + a\sin\vartheta)^2 + (b)^2}$$

Where p is the horizontal length of the rotor (m), q is the length to base of the centre (m), θ is the angular deviation from 90°, L is the average height of the suspension, a is the vertical position from centre (m).

A well plate can be used for the last stage of the mimicking process which allows a high throughput of a large range of conditions at once (Tait *et al.*, 2009). However use of the Sigma approach for scale down can result in significant differences in performance as different centrifuge design are often employed during scale up, sometimes resulting in a large reduction in Q/Σ (Hutchinson *et al.*, 2006).

1.5.2 USD Filtration

A range of ultra scale-down filtration operations have been described including microfiltration, ultrafiltration (Brou *et al.*, 2003), diafiltration (Ma *et al.*, 2010) and depth filtration (Belfort *et al.*, 1994), (Pampel *et al.*, 2008). These ultra scale-down methods are achieved using rotating disc filter designs (RDF).

The RDF is a non-linear approach to the prediction of large- scale filtration performance. Correlation between shear rate on the membrane surface and the disc rotating speed as well as the correlations between the wall shear rate on the membrane surface and inlet flow rate in lab scale cross flow cassette systems have been established (Ma *et al.*, 2010). Typically a rotating disc is placed ~1 mm from the membrane (sample material from the large scale membrane), which generates controlled levels of shear across the surface of the membrane.

A correlation was established between shear rate and RPM for the disc device, valid for cases when the viscosity is constant during the operation. Therefore the correlation does not apply to dead end more filtration

$$\gamma = a\mu^{b} RPM^{1.5} \tag{1.11}$$

Where γ is the shear rate (s^{-1}), μ is the viscosity (Pa.s), a is an experimentally determined constant and b is also an experimentally determined constant.
In tangential flow more at lab scale, shear rate at a given time is dependent on the flow rate (Q) and any additional viscosity increase is due to increase in cell concentration and the channel pressure drop.

$$\gamma = \frac{6Q/A}{\left(\frac{KQ\mu L}{\Delta Pw}\right)^{1/3}} \tag{1.12}$$

Where Q is the flowrate (ms^{-1}), A is the membrane area (m^2), K is a dimensionless coefficient, μ is the specific viscosity (Pa.s), L is the channel length (m) and ω is the effective channel width (m). Bouzerar, *et al.* (2000) pointed out that although the average shear rate is RPM dependent, the shear rate that cells are exposed to inside the RDF depend on their position with respect to the disc diameter (Bouzerar *et al.*, 2000).

$$\tau_{wt} = 0.77\mu^{1/2}(k\omega)^{3/2} r \tag{1.13}$$

Where K is the velocity entrainment factor, ω is the disc angular velocity and τ_{wt} is the wall shear stress (Pa). Bouzerar *et al.* (2000) also established that flow can be laminar near the centre of the disc and turbulent near the periphery. Therefore in order to avoid differences between in flux behaviour in a USD and a lab scale system, two key considerations are applied:

1. Shear stress and rate is dependent on the distance from the disc centre, where γ=0, unlike the lab scale system, where the shear stress is uniformly distributed over the membrane area.

2. Flux differs depending on the distance from the centre, unlike in the lab scale system, where flux is more uniform.

Overall it is clear that there are available methods to be implemented as tools for scaling down conventional primary recovery operations. These principles can be implemented during the research in order to maximise the efficiency of the experimental work. However in each case reliable scale up data will have to be shown as some ultra scale-down methods can yield poor results. For example using a small area for determination of depth filter performance may not be truly representative of the large scale operation due to inconsistency in the filter media which resulted from the initial manufacturing process.

The final introductory section will discuss the economic considerations which apply to the bioprocessing area. In particular, unit operation cost assessment methods will be discussed, including cost modelling methods.

1.6 Economic Considerations

Economic modelling in the biotechnology industry has focused on addressing cell culture decisions (e.g., selecting between fed-batch and perfusion processes) (Lim *et al.,* 2005; Pollock and Farid 2013), and chromatography operations (Stonier *et al.,* 2012, Pollock *et al.,* 2013b; Allmendinger *et al.,* 2014). Modelling tools for addressing the choice between stainless steel and single use technologies have also been reported (Farid *et al.*, 2005; Sinclair 2008). In comparison, there are few examples addressing the primary recovery area (Felo *et al.*, 2013; Pegel *et al.*, 2011).

Cost modelling in the biotechnology industry has been carried out using a variety of modelling options. Depending on the scope and the complexity of the model a choice between a static and a dynamic model can be made. Static models are faster to build and provide a basic comparison for a small number of worksheets. These have been applied to generate process cost estimates (Farid 2007; Paz and Puich *et al.,* 2004; Shaklin *et al.,* 2001;). Dynamic models are more suitable for complex scenarios, capable of encompassing parallel events as well as discrete event simulation e.g., impact of manufacturing delays, resource constraints, etc. (Banks, 1998; Paz and Puich *et al.,* 2004 Pollock *et al.,* 2013; Stonier *et al.,* 2012; Stonier *et al.,* 2009). A deterministic model is suitable for clear cut scenarios involving a limited number of inputs as it is based on carrying out a sensitivity analysis and measuring impact on key outputs by introducing an *x*% change to the input variables (Farid *et al.,* 2005; Lim *et al.,* 2006; Biwer *et al.,* 2005). Accounting for more complex and random events such as risk adjustment, is most commonly done by using Monte Carlo simulations (Hayes and Wheelwright, 1984).

A range of commercial software packages is available which provide cost modelling options including SuprerPro Designer (Intelligen Inc, Meryland, USA) and BioSolve (Buckinghamshire, UK). These offer benefits in terms of graphical representation and process design as well as ability to carry out material balances, equipment sizing and economic evaluation in a speedy manner. However novel technologies may not be captured by these packages and they do not allow incorporation of empirical models into the evaluation.

Due to the scope of this thesis and the use of alternative technologies in combination with empirically derived data a static deterministic model will be most appropriate for direct comparison of recovery unit operations. Key costs to be accounted for in the model tend to fall into two categories: capital investment required and production costs.

1.6.1 Capital Investment

Capital investment is often considered as the capital required to build a new production facility, including construction, validation and licencing. When calculated for a new build

facility this generally includes equipment costs, utility costs, piping and instrumentation costs, as well as HVAC systems and costs associated with validation, design, engineering and licencing. Previous studies have described typical capital investment costs in the range of £2 M to £750 M required to build a new facility, with typical times in the range of 2-4 years required for building and validating. The most representative method for estimation of capital investment has been suggested to be achieved by using factorial estimates which are based on previous similar projects. The Lang factor (Lang 1948) is often used in conjunction with calculated equipment costs to estimate capital investment where values in the range of 3.3-8.1 have been used for stainless steel biopharmaceutical facilities and values up to 23.7 for single use biopharmaceutical facilities (Farid 2007; Novais *et al.,* 2001; Pollock *et al.,* 2013)

1.6.2 Cost of Goods (COG)

Cost of goods (COG) generally include the operating costs associated with the production of a product. COG can be broken down into direct (e.g. raw materials, utilities etc.) and indirect costs (equipment and facility depreciation, equipment maintenance etc.). Labour costs can be included in either category, however is most commonly included in the direct costs as an hourly rate related to the processing time. The impact of each of these cost factors in mammalian cell processing have been described by Farid (2009). For example, an increase in annual production has been shown to decrease the COG/g, while material costs begin to dominate compared to the labour and indirect costs. Therefore reduction in material costs at large scale will significantly impact the COG/g, especially in downstream operations (DSP) as DSP cost has been shown to increase significantly with annual production (Farid 2009). This pattern is typically described as a downstream bottleneck and has been exacerbated by the increase in cell culture titre (Langer, 2012).

Although the trends in USP and DSP COG have been well described for mammalian processes, isolating the impact of trends in mammalian cell bioprocessing on individual processing steps is less available. Therefore creating an economic model to look specifically at COG in primary recovery operations would be necessary in order to fully describe the impact of any proposed changes to the current platform.

1.7 Aims and Thesis Structure

Published literature discussed in the previous sections has shown a continuing demand for improvements in primary recovery operation efficiency. It also highlighted that limited data is available on the future strategies for the area. This thesis aims to develop a robust methodology for the identification and selection of a future-proof primary recovery technology for the harvest of high cell concentration mammalian cell culture in mAb processing. In order to achieve this, the work will cover the identification of industry needs, creation of representative test material, primary recovery technology screening as well as detailed performance and economic assessments. This section details the aims and structure of the individual results chapters included in this thesis.

The first step in the identification of potential future proof technologies for primary recovery was assessing the current and future needs of the industry. The work described in **Chapter 3** of the thesis aimed to identify the current and future demands on primary recovery operations by surveying expert opinion across the key processing areas on the current and expected trends in cell culture, primary recovery and purification. This will identify numerical values for the worst and best case conditions, representative of the potential future and current cell culture feed profiles. The chapter results will also aim to collate the industry desired criteria for the performance of the future primary recovery technologies in order to identify the optimal assessment criteria for the technology selection process.

Chapter 4 aimed to describe a novel methodology for the generation of cell culture test material (CCTM) which will allow the test conditions identified in **Chapter 3** to be achieved. This methodology will aim to allow independent control of key cell culture variables in order to decouple the resulting effects. The methodology will aim to produce test materials for the following studies involving the testing of primary recovery technologies described in this thesis. It will be designed for the generation of a greater level of information, in turn allowing empirical correlations to be derived that link cell culture variables to the performance of primary recovery technologies tested.

Chapter 5 aimed to describe the use of CCTM to screen current and alternative primary recovery technologies, identified to have the potential to cope with predicted future feed profiles identified in **Chapter 3**. The technologies will be evaluated based on the following performance criteria: solids removal, yield and impurity removal. Selected current and alternative technology candidates will then be ranked using a multi-attribute decision-making (MADM) technique and the successful candidates will be assessed further using an economic evaluation and facility fit criteria.

Chapter 6 aimed to assess the performance of the successful technology candidates identified in **Chapter 5,** as a function of key cell culture variables. The chapter will aim to identify the most cost effective technology option for each cell concentration and product titre combination expected over the next decade in order to allow for the formation of a technology strategy for given aims of cell culture performance in terms of cell concentration and titre combinations.

The following chapter (**Chapter 2)** will describe the general materials and methods used in the course of this work. The materials and methods included in this chapter will cover detailed descriptions of the general techniques applied. Each results chapter will also include individual materials and methods sections which will focus on describing the chapter-specific experimental set up and methods used to generate the data in each chapter.

Chapter 2: Materials and Methods

2.1 Introduction

This section provides a description of the general methods and procedures used to obtain the results discussed in this thesis. The experimental designs adopted are discussed in the corresponding chapters and reference made to the relevant procedures described below.

2.2 Cell culture

Cell culture material was generated using an IgG_4 producing GS-CHO cell line - CY01 kindly provided by Lonza Biologics Plc (Slough, UK). The cell line was cultured at 5 L (bench) and 70 L (pilot) scales using stirred tank reactor (STR) systems in order to produce a cell culture material representative of commonly used large scale cell culture operations. The following sections describe the general methods used for cell recovery using the selected scales and systems.

2.2.1 Cell Line Recovery and Propagation

A working cell bank was created at passage number 8 and was stored at a concentration of $10x10^6$ cells/mL in 10% DMSO in liquid nitrogen storage. The cells from this working cell bank were used throughout all of the experimentation described in this thesis. When required a vial was removed from the liquid nitrogen store and thawed at 37°C using a water bath. The contents of the thawed vial (~$10x10^6$ cells) were added to 9mL of sterile CD-CHO (Invitrogen, Paisley, UK) medium in a 50 mL falcon tube. The cells were washed by gentle mixing with the medium then centrifuged at 450 g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 20mL of fresh sterile medium and cultured in a 125 mL shake flask for 3 days, incubated (Sanyo, Loughborough, UK) at 37°C and 5% CO_2. The condition of the cells was checked one day after recovery from frozen stock to ensure that the cell viability was above 50%. If this was not the case, the wash step was repeated to remove any remaining DMSO and the culture was continued.

Cell passages were carried out every 3-4 days. Cell number and viability was quantified using the ViCell™ (trypan blue exclusion) and cells were seeded in a new flask at $2x10^5$ cells/mL using fresh CD-CHO medium containing 0.1% methionine sulphoximine (MSX).

2.2.2 Cell Culture at the 5 L Scale

Cell expansion was carried out using 1 L shake flasks where cells were cultured for 4 days prior to the inoculation of the 5 L (3.5L working volume) stirred tank bioreactor (STR). This had an in-built control system (B. Braun BIOSTAT B-DCU control unit, Sartorius, Epsom, UK). 3 L of CD-CHO media was pumped into the reactor the day prior to inoculation in order

to preheat the media and carry out a two point calibration of the dissolved oxygen probe and a one point calibration of the pH probe using an offline pH reading.

A cell count of the inoculum was carried out on the day of the inoculation. Inoculum volume was calculated to achieve a final inoculation density of $2x10^5$ cells/mL. The inoculum as well as any remaining media required to achieve the total working volume was pumped into the reactor once the set point conditions were stable, as determined by the online DO, temperature and pH probes.

Set points were maintained at 30% air dissolved oxygen tension (DO), 7.10 pH using CO_2 and 0.1 M base and a temperature of 37°C. A constant gas flowrate of 100 cm^3/min was maintained using a horseshoe sparger. Agitation was set at 260 RPM using a single 45° pitch, three blade impeller. All cell cultures were carried out in fed-batch mode using chemically defined medium (CD-CHO, Invitrogen, Paisley, UK). The glucose concentration was measured daily with a NOVA Bioanalyser (Nova Biomedical, Deeside, UK) and maintained at a concentration of 2 g/L using a bolus feed of 10-fold concentrated dry powder CD-CHO media, adjusted to a concentration of 150 g/L glucose (Sigma, Poole, Dorset, UK). The culture was typically run to a maximum of 14-15 days.

2.2.3 Cell Culture at the 70 L Scale

Shake flask pre-culture was carried out in 1L shake flasks for 3 days and used to inoculate a 5 L STR (3.5L working volume). The inoculation procedure followed is as described in section 2.2.2 and the inoculum was expanded for a further 4 days.

45 L of CD-CHO media was made up using de-ionised grade water from powder stock of CD-CHO AGT™ (Invitrogen, Paisley, UK). Two days prior to the inoculation of the 70 L single-use bioreactor (SUB) (BIOSTAT CultiBag STR, Sartorius Stedim, UK), the single use bag was installed and inflated as per manufacturer's instructions. A Millipak 200 Gamma Gold 0.22 µm sterile filter (EMD Millipore,UK) was welded onto the media line using a BioWelder™ (Sartorius Stedim, UK). The media was then pumped into the bag at a flowrate of 100 L/h using a peristaltic pump (Watson Marlow, UK). Once the media transfer was complete, the temperature control was set to 37°C, the impeller speed was set to 150 rpm and air was pumped in at a flowrate of 2 L/min. The bag was left to soak for one day as per manufacturers instructions, at which point the dissolved oxygen optical sensor was then calibrated using air and nitrogen. The pH was manually brought within the range of the pH optical sensor using offline measurements taken with a SevenEasy pH meter (Mettler Toledo, UK) before the pH control was activated with a set point pH of 7.1 with a deadband of 0.05. Set points were maintained at 30% dissolved oxygen and a pH of 7.1 at 37℃ for one day before inoculation, and then maintained throughout the culture. Agitation rate was maintained at 150 RPM unless otherwise stated for specific experiments. The feeding

strategy was matched to the 5 L STR condition where the glucose concentration of 2 g/L was maintained using a bolus feed of 10-fold concentrated dry powder CD-CHO media, adjusted to a concentration of 150 g/L glucose (Sigma, UK). The culture was run as required, typically to maximum of 14-15 days.

2.3 Primary recovery

2.3.1 USD Centrifugation and Shear Studies

A rotating disk shear device developed at University College London was used to mimic the shear encountered in the feed zones of hermetically and non-hermetically sealed disc-stack centrifuge. Detailed methodologies for ultra-scale-down (USD) centrifugation have been described previously (Boychyn *et al.*, 2004; Hutchinson *et al.,* 2006; Levy, 1999). A microwell plate-based method described by Tait *et al.* (2009) which is a derivative of the USD method was used for these experiments.

Rotating Disc Device

A rotating shear device was used to mimic the shear experienced during the centrifugation step. Hutchinson *et al.* (2006) previously estimated the high and low energy dissipation rates equivalent to non-hermetic and hermetically sealed disc-stack centrifuge feed zones as 0.37×10^6 Wkg^{-1} and 0.019×10^6 Wkg^{-1} respectively. The methodology was used as a first step to mimic large scale centrifugation as well as for shear studies when comparing cell culture material. The rotating shear device stainless steel chamber used had a diameter of 50 mm, height of 10 mm and the rotating disc was 40mm in diameter and had a thickness of 1 mm. The shear device was filled slowly with the cell culture material using a 50 mL syringe; excess material was used to flush out any air bubbles trapped in the chamber. The selected shear was then applied to the material for 12 seconds. The material was removed from the chamber and the set condition was repeated using fresh cell culture material as required after cleaning the device with water by filling and running it up to 3 times.

Centrifugation

This part of the method was used to achieve equivalent solids separation when mimicking pilot scale centrifugation conditions. 1 mL of the sheared material prepared using selected shear conditions was transferred to rows A and H of a deep square microplate (Fisher Scientific, UK). The plate was centrifuged at 3,000 RPM for 5 minutes in an Eppendorf 5810 R bench top centrifuge (Cambridge, UK) with an A-4-62 swingout rotor. The centrifugation conditions used were set to give an equivalent feed flow rate of 100 L/h in the centrifuge to represent a moderate flowrate into a medium scale centrifuge - the CSA-1 (Westfalia, Oelde, Germany) with a sigma (Σ) value of 680 m^2. After the centrifugation was complete 500 µL of

the supernatant was carefully collected from the top of the wells, to avoid disturbing the sediment. The supernatant OD was measured and the samples were pooled for further analysis or depth filtration experimentation. Any supernatant material stored for further depth filtration experiments was stored at 4°C overnight. Samples for HPLC, BCA and DNA analysis were stored at -20°C, samples for reducing 2D PAGE gels were stored at -80°C.

2.3.2 USD Depth Filtration

Depth filtration media discs of 05SP, 10SP and 30ZA media were kindly provided by 3M (Bracknell, UK). These were cut using a 0.6 mm drill piece (Tool Zone, UK) to provide a total effective membrane area of 0.28 cm^2 and inserted into a custom made manifold. The manifold design and the filtration method have been described previously by Jackson (2011), Kong *et al.,* (2010) and Lau *et al.,* (2013). The device set up consisted of a collection plate which fitted onto the vacuum block (Tecan VacS, Tecan, UK) inside a Tecan™ platform. The vacuum manifold (Tecan™, UK) which fitted on top of the vacuum block and formed a seal with the position plate which accommodated the membrane housing. The membrane was then inserted into the membrane holder which formed the bottom part of the membrane housing. The reservoir on top of the membrane housing also served as a feed tank (see Figure 2.1).

Each new membrane was flushed with water for a minimum of 2 times at a 100 mBar pressure, while water flux through the membrane was measured. The water flux tests were repeated for a maximum of 5 times until the water flux measurements became consistent (±10%) indicating satisfactory wetting of the membrane. If consistency was not achieved, the membrane was discarded, a replacement selected, and the water flux test repeated.
Once consistent water flux measurements were obtained 4mL of feed material was pipetted into the membrane housing. Pressure was then applied at 100 mBar using a vacuum. Simultaneously a liquid handling arm (Freedom EVO® liquid handling system, Tecan, UK) was set up to monitor and record the retentate volume throughout the filtration procedure, until the flux has declined to 80% of the initial value. The scale comparison of this method has been discussed previously (Kong *et al.,* 2010, Lau *et al.,* 2012).

The results were analysed based on the V_{max} methodology assuming a gradual pore constriction model:

$$\frac{t}{V} = \frac{1}{Q_0} + \left(\frac{1}{V_{\max}}\right)t \tag{2.1}$$

where V is the total filtrate volume collected over time t, Q_0 is the initial flowrate, and V_{max} is the maximum volume that can be filtered before the filter is completely blocked and the flux reaches zero.

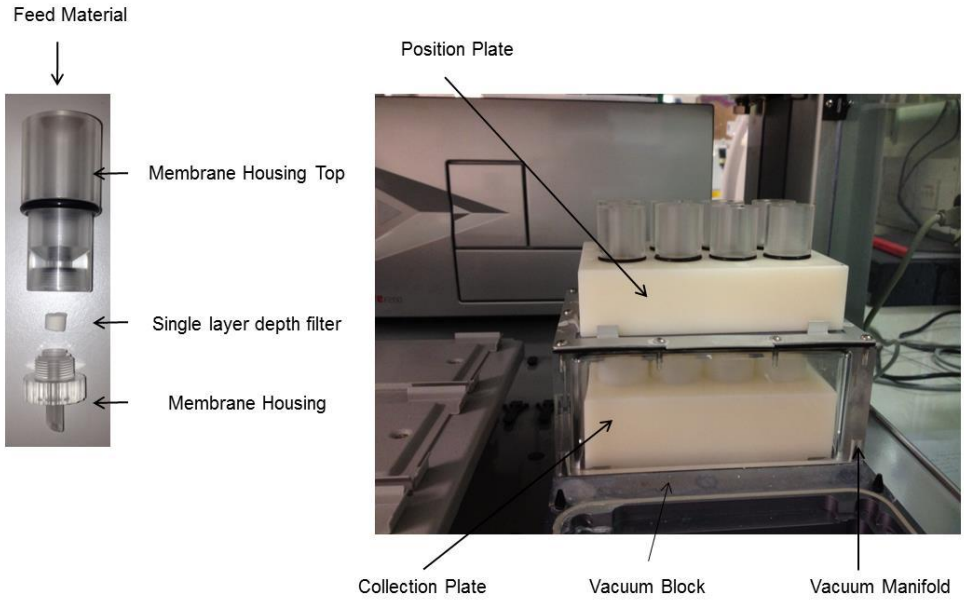

Figure 2.1: USD depth filtration manifold used for constant pressure depth filtration experiments**.**

2.3.3 Tangential Flow Filtration

Bio-Optimal MF-SL™ and QyuSpeed D™ (QSD) (Asahi Kasei, Japan) hollowfibre modules with areas of 0.0004 m^2 and 0.0006 m^2 respectively provided by a single fibre (15 cm height) were run at a constant flux of 30 LMH using an AKTA Crossflow device (GE Healthcare, Little Chalfont, UK). The initial feed flowrate was set to achieve a constant shear rate of 2,300 s^{-1} for both module types, and the backpressure was maintained positive by using a manually operated valve when required. The module was wetted for 30 minutes prior to the start of the filtration using purified water followed by 5 minutes using PBS buffer (Life Technologies, UK). Re-circulation was then stopped and the buffer was replaced with the relevant feed stock. The lines were left full with PBS prior to re-starting the circulation of the feed, reducing the liquid-air interface in order to minimise additional shear. Feed material was circulated at 2,300s^{-1} for 2 minutes prior to starting the permeate pump. Inlet, and permeate pressures were recorded using an AKTA Crossflow device (GE Healthcare, UK). Outlet pressure was recorded using a custom made pressure sensor built in UCL. The pressure sensor was used for pressure ranges between 0-2 bar and was calibrated using the AKTA Crossflow inlet pressure sensor. Permeate was collected in 15 mL graduated falcon tubes (VWR, UK), pre-weighed to 2 decimal places using a balance (Sartirus Stedim, UK). Approximately 2 mL fractions of the permeate were collected in this manner. Weights before and after filling were compared to obtain a more accurate volume of permeate in each fraction. Permeate pressure was kept above 0 by using a manual retentate valve. The operation was run until maximum recommended system pressure of 2 bar was reached. At

this point retentate volume was recorded and feed, retentate and permeate samples were collected and stored at -20°C for further analysis.

2.4 Analytical Tools

2.4.1 Cell Analysis

During the course of the cell culture and primary recovery experiments cell concentration and viability, cell size and metabolite concentrations were monitored. In addition, an apoptosis assay was also carried out.

2.4.2 Cell concentration and Viability

Cell concentration and viability were measured using a Vi-Cell XR (Beckman Coulter, UK). The method is based on imaging of trypan blue exclusion of the viable cells. When necessary, sample dilutions were carried out using calcium and magnesium free PBS solution (Life Technologies, UK) to ensure the reading was within the detection range of the device.

2.4.3 Particle Size Measurement

Cell size during the course of the cell culture as well as pre and post primary recovery operations was determined using a CASY™ analyser (Innovatis, Germany). A 150 µm capillary orifice was used to measure the number of particles which fells in the 2.5-40 µm in diameter range. A minimum of 5 measurements were carried out per sample, where any deviations in particle size distribution greater than 10% were discarded and the measurement repeated. An average of the measurements for each sample was recorded. Sample dilutions of 10-50 µL in 10 mL of Casyton™ buffer (Innovatis, Bielefeld, Germany) were used.

2.4.4 Apoptosis Assay

A commercially available Annexin V-FITC/7ADD kit (Beckman Coulter, UK) and a Coulter Epics XL-MCL Flow Cytometer (Beckman Coulter, UK) were used to determine the extent of apoptosis during the development of the cell culture test material methodology. The assay used Annexin V conjugated with fluorescein isothiocyante (FITC) and 7-amino-actynomycin D (7-ADD). Apoptosis causes the phosphatidylserine (PS) phospholipid in the inner leaflet of the plasma membrane to become exposed allowing Annexin V to bind to it (van Engeland *et al.,* 1998). The 7-ADD marker binds to DNA guanine and the cytosine base pair therefore allowing the measurement of cellular DNA when it is released into solution. The collected fluorescence data was analysed using EXPO 32 ADC XL Color software (Beckman Coulter, UK). Triplicate samples from each condition were stained and measured.

2.4.5 Solids Removal

The percentage solids removal post primary recovery operations was calculated based on optical density (OD) at 600nm measurements of the feed solution - F_{OD}, relevant sample post primary recovery - S_{OD} and was normalised to a maximum primary recovery performance achieved by passing a clarified sample from each technology additionally through a 0.2 µm PES syringe filter (Merk Millipore, UK) - $S_{100\%}$, as per equation 2.2. The OD measurements were obtained using a Thermo Biomate spectrophotometer (Thermo Scientific, UK).

$$S = \frac{F_{OD} - S_{OD}}{F_{OD} - S_{100\%}} \tag{2.2}$$

2.4.6 Solids Removal Quantification by Dry Cell Weight

In cases where the OD of a post primary recovery sample was not possible to determine, solids content was determined by measuring dry cell weight. Eppendorf tubes were weighed using a 5 decimal place balance (Sartorius Stedim, UK). Each Eppendorf was filled with 2mL of the relevant sample, including feed material, post primary recovery material and post primary recovery material which has been additionally passed through a 0.22 µm syringe filter. Triplicates of each sample were aliquoted. The samples were incubated at 80°C for 24 hours. Dry cell weight was measured and percentage solids removal during primary recovery was calculated using equation 2.2.

2.4.7 Impurity Qualification

DNA and HCP impurities were quantified post the impurity concentration stage as well as in feed samples and post primary recovery stages. DNA concentrations were determined using a Quant-iT™ PicoGreen ® dsDNA Reagent Kit (Invitrogen, Paisley, UK). The BCA assay (Fisher Scientific, Loughborough, UK) was used for quantifying relative HCP removal. HCP concentrations were calculated using the following equation:

$$[HCP] = [TP] - [TY] \tag{2.3}$$

where TP is the total protein concentration determined by the BCA assay and TY is the total product concentration in the sample, determined by HPLC analysis, described in section 2.4.8 below.

2.4.8 HPLC

IgG_4 and IgG_1 concentrations were determined using a 1mL HiTrap™ Protein G column (GE Healthcare, UK) run on an HPLC system (Agilent Technologies, UK). 100 µL of sample was loaded at less than 2 g/L at a flowrate of 2mL/min. Sodium phosphate equilibration buffer (10 mM NaH_2PO, 10 mM Na_2HPO_4, adjusted to pH 7.0) and a glycine elution buffer (20 mM, adjusted to pH 2.8) were used. Detection was carried out at 280 nm and the sample concentration was determined by integrating the elution peak and generating a standard curve using known quantities of IgG_1 or IgG_4 depending on the sample tested.

2.4.9 2D PAGE

Samples pre and post primary recovery were treated using a 2D Clean- Up Kit (GE Healthcare, UK) as per manufacturer's instructions. 200µg of protein dissolved in rehydration solution was then loaded onto a 7cm IPGPhor strip pH3-10 Non-Linear (GE Healthcare, UK). The strips were then immediately transferred to be run in the second dimension. The second dimension was run using 4-20% pre-cast Bis-Tris gels 7.0 x 7.0 x 0.1 cm ZOOM IPG Well (Life Technologies, UK) using the X-Cell SureLock Mini-Gel System (Invitrogen, UK). The gels were stained using Sypro Ruby™ (Life Technologies) stain according to the manufacturers protocol and scanned using a Typhoon 9400 laser scanner (GE Healthcare, UK) with a 100µm pixel size and 600 V PMT. The images were analysed using SameSpots software (TotalLab, UK). Normalised spot volumes were calculated and compared across the gels.

Chapter 3: Defining targets for mammalian cell culture primary recovery operations in the future by quantifying current industry trends

3.1 Introduction

Key variables of a typical mammalian cell culture feed to primary recovery, which impact the performance of primary recovery and purification operations include cell line and process specific parameters: cell concentration, cell culture titre and cell viability at harvest (Tait, *et al.,* 2009; Westoby *et al.,* 2001). In turn, these affect the impurity profile present in the feed streams to the purification operations, including DNA and HCP concentrations. As a consequence final product quality, purity and yield.

Due to the current reported constraints in primary recovery changes are impacted in the technologies implemented for manufacture may be required. In order to ensure a future proof, robust choice of primary recovery technology, it is important to define the expected worst case conditions over the envisaged time period for technology implementation. Typically, purchased equipment is expected to be utilised for a period of at least ten years and therefore the time period considered in this study accounted for this. Accordingly the likely values which cell concentration, titre, cell viability and impurity concentrations might be expected to reach over the next decade will be defined in this chapter. To carry out this definition, it will be necessary to consider the relationship between these process parameters.

3.1.1 Titre Increases

Overall, titre increases can be achieved through increases in both cell specific productivity and in cell concentration which in turn can be manipulated through cell line engineering (Tiggers and Fussenegger, 2006) and cell culture process optimisation (e.g., feeding strategy, cell culture mode etc.) (Jiang and Sharfstein, 2007). Titre increases achieved by process optimisation can potentially be characterised by carrying out a practical investigation quantifying the impact of bioreactor operation conditions on titre and its relationship with cell concentration, specific productivity, and impurity concentrations. However, predicting the outcome of current research in cell line engineering requires not only the collation of the progress made to date in cell line engineering found in literature, but more specific knowledge of current industrial research, aims and methodology as well as industrial experience to date. This information is mostly unavailable to the public and is limited to industrial experts working in the field. Current process development implements a combination of cell line engineering and process optimisation (Huang *et al.,* 2010).

3.1.2 Cell concentration

Cell concentration is generally optimised through bioreactor operation after the clone selection process has been accomplished (Todaro and Green, 1963). An increase in cell concentration achieves an increase in titre, however it can also result in an increase in % solids concentration, as well as the potential HCP and DNA concentrations, due to the increase in the number of cells present. This can also result in an increase in viscosity, especially at low cell viabilities, due to the release of cellular contents into solution.

3.1.3 Cell Viability

Cell viability at the point of harvest is generally controlled. Low viability harvests are not typically recommended (Iammarino *et al.,* 2007). Shear, which is typically present during primary recovery operations, results in a greater degree of cell breakage when processing low viability cell broth (Tait, 2009). Cell breakage in turn results in an increase of HCP and DNA concentrations in the subsequent processing streams, which increases the burden on downstream operations to achieve the required level of impurity removal. For example; a high cell concentration, low viability feed stream composition provides challenging conditions for primary recovery and purification operations.

This chapter aims to identify the current and future demands on primary recovery operations by surveying expert opinion across the key processing areas on the current and expected trends in cell culture, primary recovery and purification. This will allow the identification of numerical values for the worst and best case conditions representative of the potential future and current cell culture feed profiles. In addition, the survey aims to collate information regarding the desired criteria for the performance of the future primary recovery technologies in order to identify the optimal assessment criteria the technology selection process.

3.2 Materials and Methods

A survey was constructed which consisted of 13 questions covering topics concerning cell culture primary recovery and purification as well as regulatory issues (Appendix A). Each answer option required a confidence rating of low, medium or high, where low confidence corresponded to an answer based on low, medium confidence was based on general previous experience and high confidence was based on specific data or experience.

In order to increase the quality of the data gathered, the participants were carefully selected by recommendation as industry experts. The survey was carried out in a contract manufacturing organisation, as it had a broad range of mammalian cell processing experience due and therefore a good understanding of processing requirements and trends. A sample group of 16 experts across the processing areas were selected by internal company recommendation to complete the survey, which was distributed via email. Six of the experts were also selected for additional 30 minute interviews, in order to expand on the answers they provided and to cover the topics in greater depth.

The experts included technical experts from upstream, downstream and analytical groups. As primary recovery operations fall between the upstream and downstream areas both points of view were captured in order to draw conclusions allowing for experimental design definition and criteria setting. The experts were also from different groups including process development and manufacturing development in order to capture any difference in the trends observed by the two groups.

The survey results were analysed by incorporating an appropriate numerical value to the corresponding confidence rating as outlined in Table 3.1. Question 1 was designed to identify the key operational areas which have experienced constraints over the past 2 years. Question 2 aimed to determine whether bioprocess developers have become more open to the use of alternative technologies, as technology uptake can impact technology choice made by bioprocess developers. Questions 3-5 aimed to determine the cell concentration and product titre range that the experts were expecting to reach in the next decade. Question 6 then asked to specify which cell culture technology was expected to be used to reach these cell concentrations. As discussed in section 1.2.3, the cell concentration ranges are highly dependent on the technologies selected (e.g., perfusion technology may be used to reach up to $300x10^6$ cells/mL, whereas fed batch culture technologies currently achieve cell concentrations in the range of $20\text{-}50x10^6$ cells/mL). Questions 7-8 focused on collecting additional information regarding current processing constraints and the cell concentrations under which they have been experienced. Question 9-12 aimed to quantify the current desired features for primary recovery operations in terms of impurity removal. Finally, question 13 aimed to quantify expected changes in the regulatory guidance or product

release specifications in order to identify if any additional pressure on unit operation performance will be expected in the future.

Table 3.1: *Confidence ratings available to support answers in the designed survey questions and the equivalent numerical values, allowing to calculate confidence weighted result scores for survey data analysis.*

Confidence Rating Option	Equivalent Numerical Value
Low	1
Medium	2
High	3

This technique is similar to assigning priority weighting to selection criteria. Subsequently, the results were scored based on the frequency of answer and the confidence rating given as described in equation 3.1:

$$FR = f \times C_R \tag{3.1}$$

where *FR* is the confidence weighted frequency rating, *f* is the result frequency and C_R is the equivalent numerical value of the confidence rating basis allowing a numerical outcome. In cases where answer options were compared, the percentage confidence weighted frequency rating (*PFR*) was calculated as shown in equation 3.2:

$$PFR = \frac{f_a \times C_{Ra}}{\sum_{i>1}^{n} \left(f_i \times C_{Ri} \right)} \times 100 \tag{3.2}$$

Where f_a was the frequency of option *a* selected in an answer to a question, C_{Ra} was the confidence rating of the answer in option *a*, f_i was the frequency of option *i* available as an answer option, C_{Ri} is the confidence rating given for option *i* and *n* is the number of available answer options.

3.3 Results and Discussion

3.3.1 Trends in Upstream Processing

The survey was completed by sixteen industry experts in a contract manufacturing organisation in order to quantify the current and expected trends across key processing areas. The full questionnaire is shown in Appendix A. Contract manufacturing companies

tend to have high levels of experience due to the large number of products and processes they encounter and therefore are qualified to serve as a representative reflection of the industry as a whole. Although the expert sample size was relatively small, the effect on the quality of the collected data was minimised by participant selection (e.g., participants with high levels of experience), while quantifying their level of expertise using self-assessment. Six of these experts were also interviewed to provide additional clarification and detail to their answers.

In order to contextualise the processing constraints reported in literature (see sections 1.2.2 1.2.3), experts were asked to state the processing areas in which operational constraints had been experienced over the past two years. The results showed that the majority of the operational constraints between the years of 2009 and 2011 had been seen in purification (38%), followed by primary recovery (30%) and cell culture (23%) (see Figure 3.1). This is consistent with literature reports and provided the impetus for this thesis (see section 1.7). Interview results suggested that a proportion of the purification constraints were due to high impurity content feed streams, causing the precipitation of impurities during the pH hold step (for process diagram see Figure 1.1). *In situ* pH induced cell settling prior to recovery by depth filtration or a combination of centrifugation followed by depth filtration would prove beneficial in this instance, as it would eliminate the need for introducing an extra filtration train post the pH hold step, taking advantage of the existing unit operations at primary recovery. At large scales, however, *in situ* pH change may be impractical due to the volume and mixing requirements. Overall, this highlights a drive to achieve a greater degree of impurity removal during primary recovery operations.

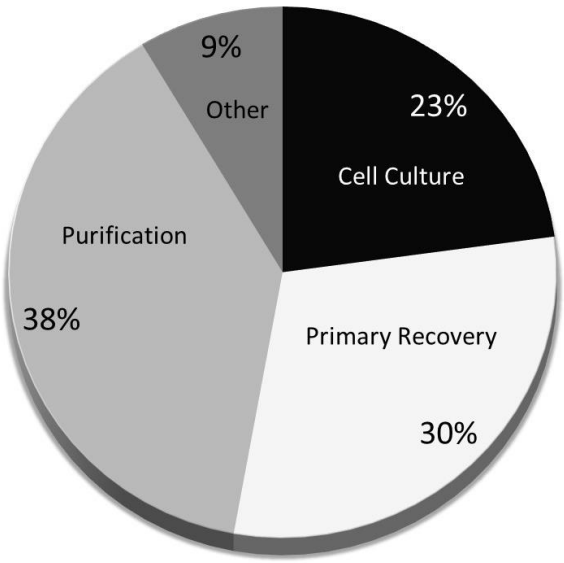

Figure 3.1: *Quantification of operational constraints experienced between 2009 and 2011 by processing area. Percentage confidence weighted frequency ratings have been shown and calculated as shown in equation 2.*

The survey participants were then asked to state the titre and cell concentration at harvest they expected to be the norm by 2020. Although no multiple choice options were given in this question to avoid limiting the participants in their answers, their responses fell into specific groups with many participants stating the same target cell concentrations and titres. Individual confidence values for cell concentration and titre were averaged to give an average confidence rating. This was plotted against the equivalent cell concentration and titre combinations to help identify the most likely combination expected to be reached within the next decade (see Figure 3.2). Expected titres ranged between 10-15 g/L but were associated with low confidence values. Titres above 15 g/L were stated with medium to high levels of confidence. The expected cell concentration range of 40-80x10^6cells/mL was frequently stated, however the average confidence rating was low (<2) with few high confidence answers. Four answers were in the 80-100x10^6 cells/mL range with confidence ranging between low to medium.

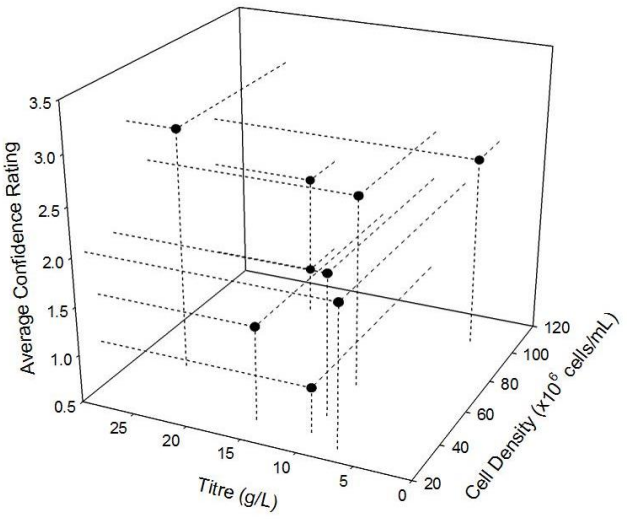

Figure 3.2: *Cell concentrations and titre expected by industry survey participants to be reached in the next 12 years. Equivalent confidence ratings given for titre and cell concentration values were averaged and plotted for each given combination.*

Areas of highest confidence included titre ranges of 10-20 g/L and equivalent cell concentrations of 25-100x10^6 cells/mL. Titres beyond 10g/L has been reported in literature as well as cell concentrations above 100x10^6 cells/mL achieved with the use of perfusion technologies (Huang *et al.,* 2010; Clinke *et al.,* 2013). Therefore the stated range can be considered realistic in light of these independent sources. However it must also be noted that a change in cell culture technology (from the current fed-batch operation) may be necessary in order to achieve realistically these targets. When asked for reasoning behind these choices in titre and cell concentration combinations, interview participants who chose high cell concentration high titre combinations defended their choice based on the previous trends and cell concentration increases seen over the years, as well as potential changes in

the cell culture technology likely to be applied in the future. Interview participants who gave lower range figures stated that their choice was based on the currently achieved higher titres and cell concentrations, which they expected to become routine.

Based on these results it is possible to state the range of expected titres and cell concentrations to be 10-20 g/L and 20-100x10^6 cells/mL respectively. This range of cell concentrations correlated with the current industry trends discussed in section 1.2.3, where cell concentrations of up to 300x10^6 cells/mL were achieved using perfusion technologies (Schirmer *et al.*, 2010; Clincke *et al*., 2013a; Clincke *et al*., 2013b; Karst *et al.,* 2016). However, cell concentrations reported using fed batch processes are in the range of 20-40x10^6 cells/mL, in line with the predictions made. These titre and cell concentration rangeswill be simulated as a potential future cell culture material in the following work.

3.3.2 Quantifying Purification Processing Area Needs

Primary recovery platform options have typically included depth filtration options or a combination of centrifugation and depth filtration (see section 1.1.2) (Kelley *et al.,* 2009; Liu *et al.,* 2010). Interview results confirmed this and provided a clear current scale definition for the application of these two options. Depth filtration is typically implemented at scales less than 1,000 L and centrifugation followed by depth filtration at scales greater than 1,000 L. In order to gauge the general industry trends and interest in alternative processing options, the survey participants were asked whether they had observed an increase in their customer interest in unit operation alternatives to the platform options offered (see Figure 3.3). 83% of the participants answered noted an increase in customer interest in alternative processing options. They were then asked to identify the processing options of interest. Protein A alternatives were the most sought after with a percentage confidence weighted frequency rating (PFR) value of 29%, followed closely by cell culture alternatives (21%) and flocculation options (12%).

The use of alternative unit operations requires additional development, knowledge base building and extensive characterisation, the extent of which depends on the currently available information on a given technology. Carrying out such work requires substantial resources and the inevitable risk associated with unknown performance output as well as potential failure in the selected mode of application. Interview results hypothesised that the difference in customer interest is likely due to the existence of two different customer types: small to medium and large enterprises (SME and LE respectively). Large enterprises are more likely to have the resources required to invest in the development and implementation of alternative options. SMEs are less likely to make the investment due to the high risk, as well as the time and cost constraints. Overall, the quantified expressed interest shows a potential demand for expanding the currently offered platform options.

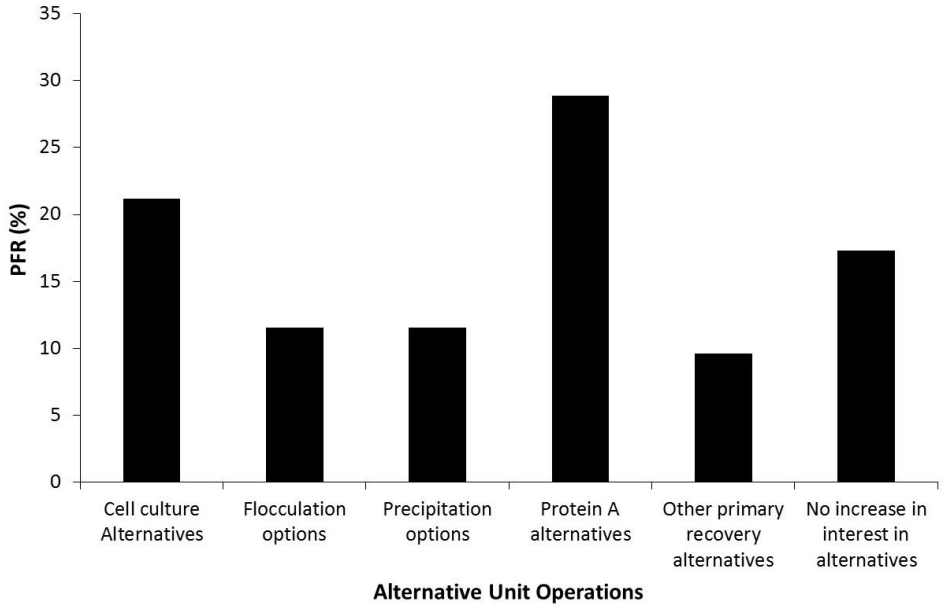

Figure 3.3: *Increase in interest expressed by CMO customers in alternative unit operations. Percentage confidence weighted frequency rating (PFR) was calculated using Equation 2.*

In order to outline the impurity removal criteria upon which technologies with the potential for future use might be selected, the survey participants were asked to select the desired level of impurity removal to be achieved during the primary recovery stages (see Figure 3.4). The impurities were categorised as host cell proteins (HCPs), product aggregates and DNA were presented in a random order. An option of "other" was also available with a space to further specify any additional desired impurities. The % removal options were as follows: 1-10%, 10-20% and 20-40% removal. 20-40% HCP removal received the highest (22%) PFR value. Desired aggregate removal levels of 1-10% and 10-20% received similar PFR values of 18% and 16% respectively. High levels of DNA removal (2-40%) received a PFR value of 15%, while the PFR value given to low and medium levels of DNA removal were 9% and 4% respectively. Although the answer to this question was aimed to provide guidance on the desired primary recovery operation features, the given answers may indicate that the participants prioritised impurity removal which would be reasonable to expect from a primary recovery technology. For example current technologies such as depth filtration options provide a higher level of DNA removal and a low level of HCP removal (Yigzaw *et al.*, 2006; Charlton *et al.*, 1999). As these options are based on non-specific selection they do not offer aggregate removal. Therefore the current technology ability is consistent with the desired primary recovery operation features, which may indicate that the expert answers were biased by the expectations they have for technology performance in the future.

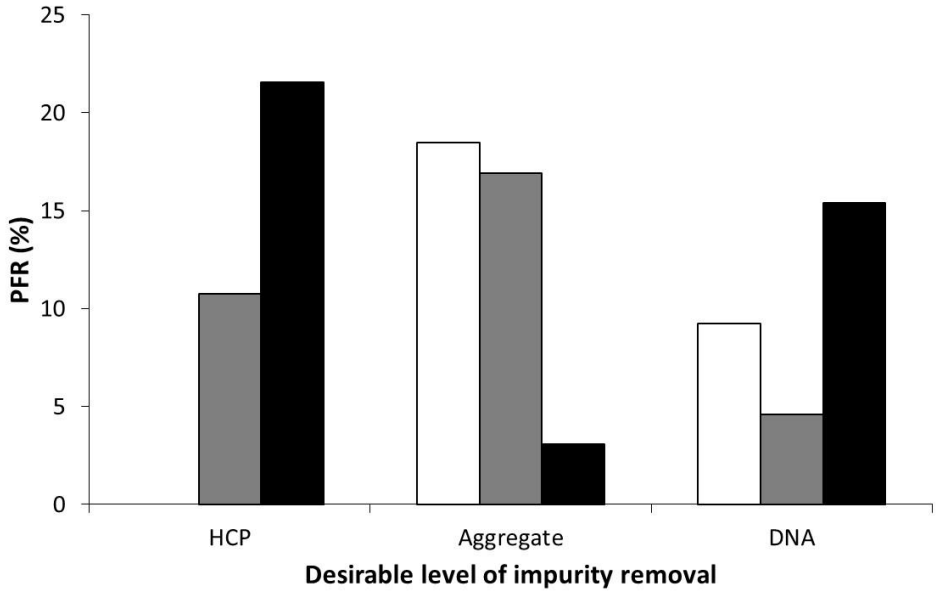

Figure 3.4: *Desired level of impurity removal during primary recovery operations quantified using the percentage confidence weighted frequency rating (PFR) calculated using Equation 2. Industry experts were asked to specify a range of HCP, product aggregate and DNA removal they would like to see achieved during primary recovery. The available impurity ranges included: 1-10%; 10-20%; 20-40%; option of "other" was also available but was not selected by any participants.*

These percentage removal results closely reflect the currently reported and advertised levels of impurity removal achieved using charged depth filtration (Lambalot *et al.*, 2006), and therefore provide potential minimum levels for future performance targets. They also provide a basis for ranking the impurities in terms of importance of removal by selecting the highest PFR figures in each impurity group and using these to order the impurity groups from highest to lowest levels of desired removal. Impurity removal can therefore be ranked in the following order: HCP, DNA, and product aggregates (see Table 3.2).

Table 3.2: *Impurity removal priority criteria in terms of HCP, DNA and product aggregate removal during primary recovery processing stages, derived by quantification of industry expert opinion.*

Priority of Removal	Impurity
1	HCP
2	DNA
3	Product Aggregates

3.4 Conclusions

A survey was carried out in order to quantify the current and expected trends in cell culture, primary recovery and purification operations. Although the significance of the results obtained are limited by the small sample size of survey participants, participant knowledge base and experience in the field was likely to result in high quality answers. The breadth of processes considered by the participants when completing the survey was also likely to be limited by the processes run by the participant company. However, as the participant company was a CMO, the experience of the survey participants was likely to include an overall industry perspective due to their knowledge of their customer requirements (i.e., global biopharmaceutical developers).

The results of the survey confirmed the trends implied from the literature, including increased operational constraints in primary recovery and purification processing areas, as well as increases in impurity loads in streams passed to purification. This suggests that impurity removal as well as solids removal are likely to be important attributes of future primary recovery unit operations.

An increase in customer interest in alternative unit operations has been observed by process. Interest in Protein A alternatives has been shown to be the most prevalent, followed by cell culture alternatives and some primary recovery alternatives. Although this increase in interest is likely to represent mostly large enterprises, due to the investment required for development and implementation of new technologies, it is an indicator of increased demand for new technologies across the sector.

Expected titre and cell concentration ranges have been identified for the future cell culture feed to primary recovery based on the average confidence weighted expert opinion. Expected titre in 2020 has been estimated to be in the range of 10-20 g/L with an expected cell concentration projected to be in the range of 50-100x10^6 cells/mL. These figures are to be used as representative ranges for the primary recovery technologies, selected on their capability to be implemented robustly in the future. Impurity removal targets during primary recovery have also been quantified as priority criteria to be used in the assessment of potential technology performance. HCP removal has been identified as the primary concern, followed by DNA and product aggregate removal.

This chapter has quantified the cell culture conditions expected to be reached in the future by providing an overview of the current expert opinion. However, these high cell concentration, high titre and impurity load conditions remain difficult to achieve in practice in order to provide representative cell culture material for experimental work. The next chapter will focus on addressing this challenge by describing a methodology for the production of cell

culture test materials aimed at achieving these high cell concentration, high titre and impurity conditions.

Chapter 4: Generating a cell culture test material (CCTM)

4.1 Introduction

The previous chapter focused on quantifying the expected ranges for future cell culture feed profile characteristics as well as the key variables affecting primary recovery operations. The results identified potential future cell concentration ranges in the order of 50-100x10^6 cells/mL and titre ranges of 10-20 g/L. This chapter will address the challenge of creating material representative of these ranges which can be used as a test material for primary recovery operations.

Current cell culture titres have been reported to have reached >13 g/L in fed-batch cultures (Huang *et al.,* 2004), with current cell concentrations beyond 20 × 10^6 cells/mL (Westoby *et al.,* 2011) (see section 1.2.3). These rises have contributed to an increase in pressure on primary recovery and purification operations, which have to adapt to changes in the feed stream. Although research into alternative downstream technology options has been carried out over the years (Liu *et al.,* 2010), there has been little focus on how to generate representative feed materials for experimentation.

The key variables, which define the properties of feeds to primary recovery operations tend to be specific to the operation in question. However, most published studies have historically included factors such as solids load (Hutchinson *et al.,* 2006), cell concentration and cell viability (Singh *et al.,* 2013), and more specifically particle size distribution (Tait *et al.,* 2009). The impact of primary recovery and purification operations on product recovery as well as DNA and host cell protein (HCP) impurity removal have been discussed (Westoby *et al.,* 2011), most often in the context of cell line and cell culture conditions (Tarrant *et al.,* 2012).

Research focused on increasing the understanding of primary recovery technologies such as centrifugation (Boychyn *et al.,* 2004; Maybury *et al.,* 2000), depth filtration (Lau *et al.,* 2013; Riske *et al.,* 2007) and tangential flow filtration (TFF) options (van Reis *et al.,* 1982), have typically used cell culture material harvested directly from bioreactors. In these cases harvest conditions have been stated, such as day of harvest, cell concentration at harvest or viability at harvest, but little evidence of control over these feedstream conditions were shown. Some studies have focused on just one of the variables, for example cell viability, by harvesting material at different days from the cell culture bioreactor to obtain feed material with varying levels of viability (Tait *et al.,* 2009). However, adopting such a methodology can make it difficult to contextualize the results when relating to other cell lines or cell culture processes. For example at identical cell culture days different cell lines or processes can yield a range of viabilities. In addition unique cell death pathways can result in differing particle size distributions being passed onto the primary recovery operations (Velez-Suberbie *et al.,* 2013).

Spiking of impurities as well as product has also been used in the past to mimic different levels of impurity loads (Kong *et al.,* 2010; Shirataki *et al.,* 2011) or to provide a measurable concentration, which can be quantified prior to and post-technology or condition implementation. However, the stability of the spiked material in solution must be verified to ensure the spiked material can be accurately quantified.

Comparing the cell culture material produced using different cell lines for primary recovery studies and purification has also been previously shown (Westoby *et al.,* 2011; Singh *et al.,* 2013). However, this method can have limitations when deriving correlations or empirical models, as potential biological expression differences can create a large set of unknowns, especially where HCP profile differences are significant.

The level of control required to achieve specific properties in cell culture feeds to downstream operations can cause considerable strain on the cell culture material generation even prior to the material reaching primary recovery stages. Other variables can also contribute to the number of unknowns which in turn could potentially affect the downstream experimentation including cell culture scale and scale up methodology, feeding strategies, and metabolite profiles during processing.

This chapter describes a novel methodology for the consistent creation of materials with a defined set of process-relevant characteristics and is aimed at producing material for the primary recovery studies described in the following chapters. The approach is to control tightly key cell culture variables and to ensure they are maintained independently so as to decouple effects from one another. Such an approach is difficult to achieve when using cell culture material sourced directly from a cell culture operation. The methodology aims to increase the level of information which can then be derived from carrying out primary recovery and purification studies and to provide a new avenue for deriving empirical performance correlations for novel processing technologies, in particular those designed to handle high cell concentration feeds.

4.2 Materials and Methods

4.2.1 Cell Culture 5 L Scale

Cell culture material was generated using as described in section 2.2.2.

4.2.2 Cell Culture 70L Scale

The CY01 cell line was also used to produce material at the 70L scale using a single-use bioreactor (SUB) (BIOSTAT CultiBag STR, Sartorius, UK). Set points were maintained at 30% dissolved oxygen and a pH of 7.1 at 37℃. Agitation rate was set at 150 RPM. Cell culture media and feeding strategy were matched to the 5L STR conditions (see section 2.2.3 for a detailed description).

4.2.3 Shear Study Comparison

Cell culture material was harvested at the 5L scale once the viability had reached approximately 75%. Samples of the material were loaded into a rotating shear device (Boychyn *et al.,* 2004; Hutchinson *et al.,* 2006; Levy *et al.,* 1999; Tait *et al.,* 2009). High and low shear (equivalent to maximum energy dissipation rates of $0.37x10^6$ Wkg^{-1} and $0.019x10^6$ Wkg^{-1} respectively) were applied to the material, with each condition examined in triplicate. Particle size distributions obtained pre and post exposure to high and low shear of each material batch were normalised and compared.

4.2.4 Cell Culture Harvest

All 5L STR harvests were performed on days 13 to 14 of culture, once the viability has declined to ~70%. The 70 L STR harvests were performed daily between day 7 and day 14 of culture, where 3L of cell culture material was removed per daily harvest.

4.2.5 Induced Cell Apoptosis

On day 13 of the cell cultures, approximately 300 mL of cell culture material was removed from the 5L STR, aliquoted into 50 mL falcon tubes and washed with a sterile phosphate buffered saline solution (PBS, Sigma-Aldrich, Gillingham, Dorset, UK). The sedimented cells were then incubated on ice for 2 hours, then maintained at 37°C for up to 24 hours prior to use. Samples were monitored in the first 6 hours post apoptosis induction by staining using the Annexin V-FITC/7ADD kit PN IM3546 (Beckman Coulter, High Wycombe, UK) and processed using a Coulter Epics XL-MCL Flow Cytometer (Beckman Coulter, High Wycombe, UK). Apoptosis data was collected and analyzed using EXPO 32 ADC XL Color software (Beckman Coulter, High Wycombe, UK). Samples were analyzed using 488 nm excitation and detected using 525 nm and 675 nm band-pass filters for Annexin V and 7-ADD (7-amino-actynomycin D) respectively, and collected for 300s (10,000 events). On the

day of harvest the required volume of the dead cell stock was calculated based on cell concentration and viability measurements of both the harvest material and the dead cell stock, obtained using a ViCell™ (Invitrogen, Paisley, UK).

4.2.6 Cell concentration

Cell culture material was concentrated post-harvest, using an Asahi Kasei Bio-Optimal MF SL™ module, area 0.005 m^2 (Asahi Kasei Medical Co., Tokyo, Japan). The module was run in TFF mode using the AKTA crossflow system (GE Healthcare, Little Chalfort, UK) as well as a manually controlled peristaltic pump for permeate flow control. The feed was circulated at 240mL/min and permeate was collected at a constant flowrate of 3.5mL/min, until the target concentration factor was achieved. The starting and final concentration volumes (V_S and V_C respectively in equations 4.1 & 4.2) were calculated based on the starting (D_S) and desired (D_R) cell concentrations, as well as the spike volumes of the product (IgG_{SP} and impurity spikes (HCP_{SP}, DNA_{SP}) and the dead cell stock spike (DC_{SP}) required (Figure 4.1).

$$V_S = \frac{D_R \times V_R}{D_S} \tag{4.1}$$

$$V_C = V_R - \left(HCP_{SP} + DNA_{SP} + IgG_{SP} + DC_{SP}\right) \tag{4.2}$$

4.1.1 Impurity and Product Concentration

Fully purified IgG_1 and flowthrough fraction post Protein A chromatography was kindly provided by Lonza Biologics. The flowthrough fraction was used to produce the HCP spike stock solution while the fully purified IgG_1 was used to create the product stock solution. Both the HCP and IgG_1 fractions were concentrated to approximately 50g/L using a Vivaspin®20 (Sartorius, Epsom, UK) with 5,000 and 50,000 MWCO filters respectively. Post treatment concentrations of the HCP stock were confirmed using the BCA assay (Fisher Scientific, Loughborough, UK). The IgG_1 was quantified by measuring absorbance at 280nm. All analytical methods applied in this chapter can be found in sections 2.4.7 and 2.4.8.

4.3 Results and Discussion

4.3.1 Analysis of cell culture test material (CCTM)

A representative set of mimic feeds to primary recovery, termed cell culture test materials CCTM were generated to create a wide range of cell culture conditions. The range of conditions generated match current cell concentration ranges as well as those reported to be high cell concentration conditions (20-100x10^6 cells/mL). The methodology used is summarised in Figure 4.1 and involved harvesting low cell concentration cell culture (<10x10^6 cells/mL) material produced in a stirred tank bioreactor and concentrating it using a tangential flow filtration step. An apoptotic cell stock was also created which may be used as a cell stock to achieve required cell viability conditions. Product and HCP stocks were also created by concentrating a GS-CHO cell line derived IgG_1 and impurities. These were used to achieve target product and impurity concentrations for the selected conditions. The resultant CCTM samples were characterised in terms of the key variables of cell culture materials when used for primary recovery studies. The methodology was analysed across the two scales used to generate the cell culture material, the consistency of the final material was also assessed.

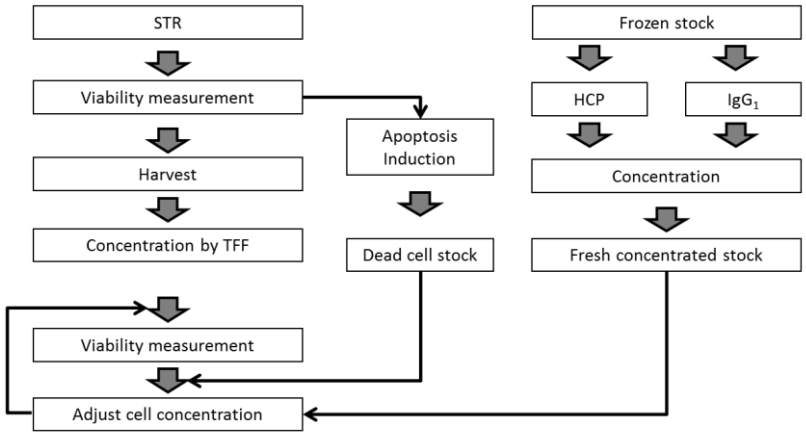

Figure 4.1: *Schematic illustrating the template methodology used to produce the cell culture test material CCTM, with independent control of cell concentration, cell viability, HCP, and IgG_1 concentrations. This methodology was used to achieve a range of high cell concentrations, viabilities, titre, and HCP concentrations by using cell culture material harvested from an STR at the 5L and 70L sales at a cell concentration <10 × 10^6 cells/mL and a titre of <1 g/L. The cell cultures were harvested and concentrated using TFF, to achieve the target CCTM cell concentration. When required apoptosis induction was used to generate a dead cell stock which when spiked was able to provide the target viability of the CCTM. HCP and IgG_1 originating from the GS-CHO cell line were spiked into the CCTM to achieve the target impurity and product concentrations.*

4.3.2 Impact of the Cell Material Source

Cell culture material was produced at bench (5 L) and pilot scales (70 L). Cell growth, viability profiles and the average specific productivity during scale up were predominantly maintained within one standard deviation of the bench scale results for the majority of the runs (Figure 4.2). However, an apparent drop in viability was detected at the 70 L scale after 250 hours of cell culture. 48 hours later the viability was once again within one standard deviation of the 5 L scale average. This inconsistency after 250 hours of cell culture is likely due to poor sampling technique, as suggested by the rapid restoration of the viability back to the expected range. This can often be caused by insufficient flushing of the sample tube prior to sampling.

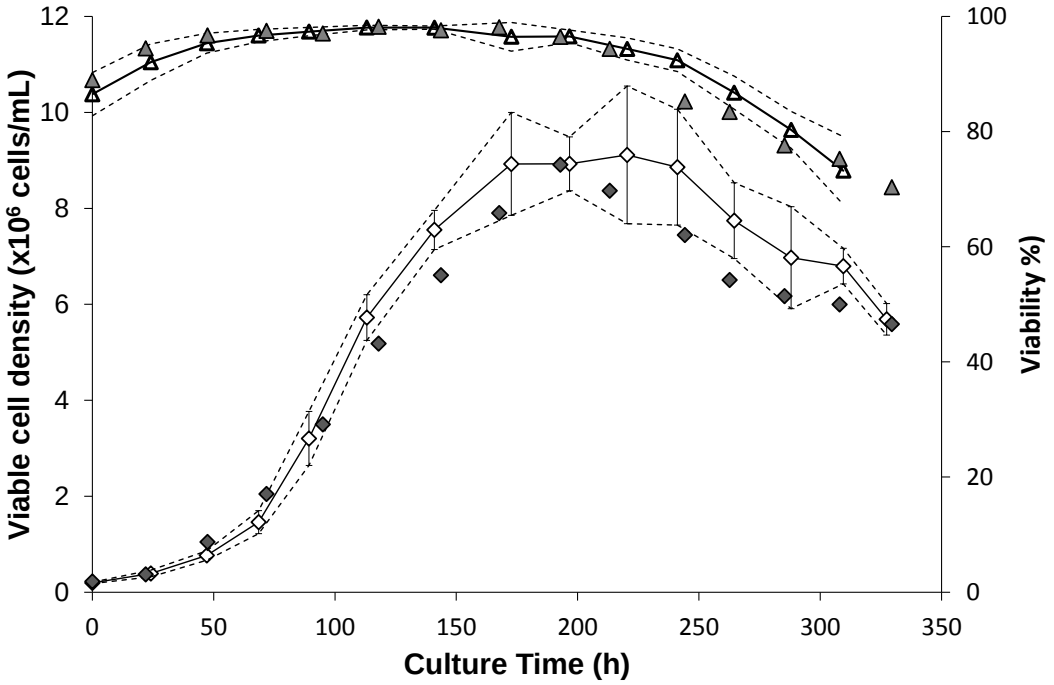

Figure 4.2: *Cell culture growth curves comparing the cell culture growth profiles achieved at two scales. Average viable cell concentration at the 5L scale calculated using data from 5 biological replicates (◇) and cell viability (△) is shown throughout a 14 day culture period. Average viable cell concentration at the 70L scale calculated using three technical replicates (◆) and cell culture viability (▲) is shown. Dotted lines above and below the average lines indicate ±1 standard deviation of the repeat 5L cultures and error bars indicate ±1 standard deviation from a single 70L scale run. NOTE: individual 5L run results are plotted in Appendix B.*

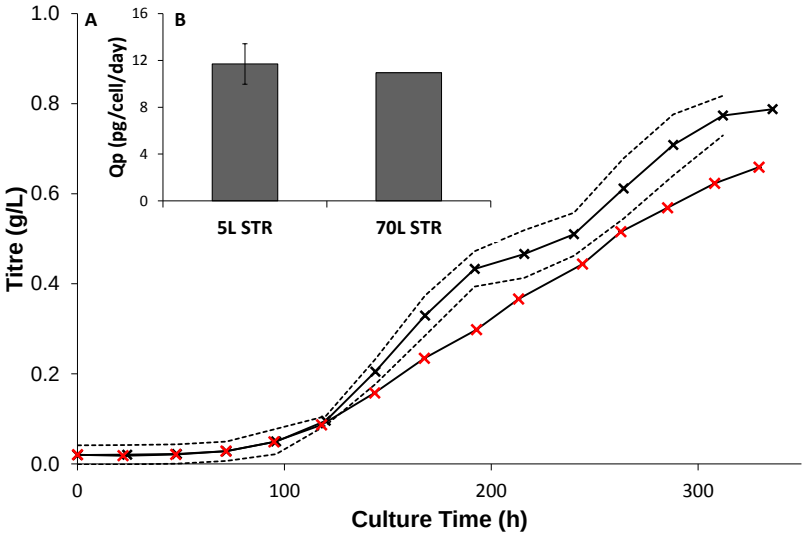

Figure 4.3A*:Cell culture IgG₄ titre curves achieved at the 5L scale, where the average of 5 repeated cell cultures is shown (x) as well as the upper and lower bounds of one standard deviation shown using the black dotted lines. Cell culture titre curve for the 70L scale is calculated using one run repeat, and an average of triplicate samples is shown (x). The error bars represent one standard deviation. B: Specific productivity calculated for five 5L scale cell culture runs compared to that obtained at the 70L is shown.*

The IgG_4 production seen at the 5L scale was comparable to that at the 70L scale between 0 and 120h. Post 120 hours the titre reached using the 5L system was approximately 20-30% higher than at the 70L scale (Figure 4.3A). However, the cell specific productivity at both scales is within one standard deviation of the batch to batch variation seen at the 5L scale (Figure 4.3B). Therefore the titre difference is due to a decrease in the integral viable cell count (IVCC) throughout the culture which translates to this small decrease in the overall titre. A decrease in the IVCC can be explained by the implementation of a single use bag at the 70L scale as opposed to the glass tank at the 5L scale. The impact of leachables in the single use system could have been the cause. Vessel material as well as other potential differences such as the volumetric power input have been recommended to be maintained during scale up (Nienow, 2013a; Nienow 2013b). Neither were maintained in this case and this may have had an effect on cell specific productivity observed in figure 4.3B.

Cell shear sensitivity profiles of the cell culture materials were also compared. This was carried out by subjecting cell culture material harvested from both the 5L STR and the 70L SUB at equivalent viabilities of ~75% to conditions of no, low and high shear (Figure 4.4A, B and C). Prior to the application of shear three populations of cellular material could be observed at both scales. The 0-7µm region showing small diameter cell debris, 7-12µm region showing apoptotic cell populations and 12-30µm region showing live cells. As low levels of shear were applied a shift towards smaller particle sizes was observed, as well as an increase in the apoptotic cell population. The decrease in cell size as a response to applied stress conditions has been observed previously (Tait *et al.,* 2009; Tarrant *et al.,* 2012). In addition cell damage is expected as shear is applied, causing some of the cells to burst, and subsequently the observed shift in cell size. The change in particle size distribution profiles between no shear and low shear yielded similar profiles at 5 and 70 L scales.

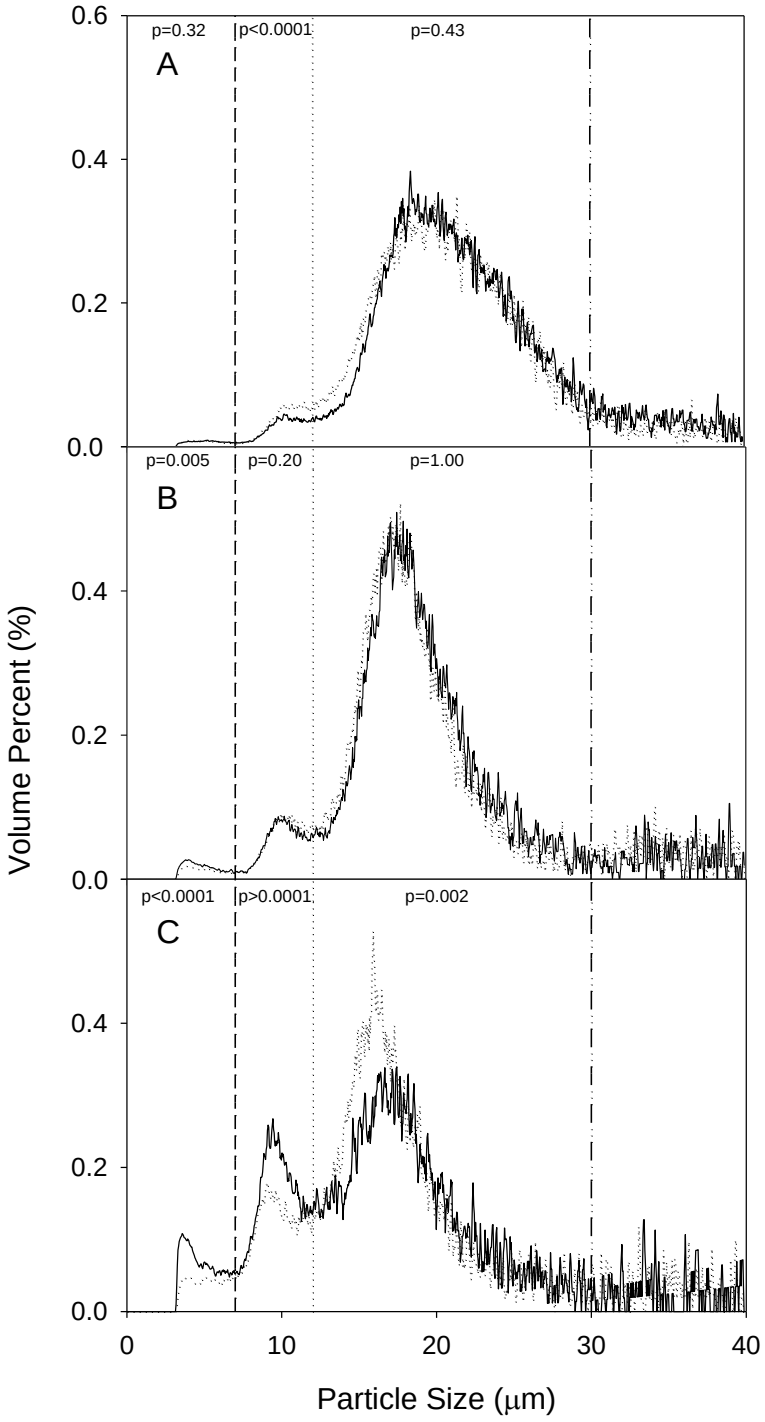

Figure 4.4 (A): *Particle size distributions of cell culture material harvested at 70–75% viability at the 5-L (black line) and the 70L (dotted line) scales prior to the application of shear. Average particle size distributions of each sample were obtained (n = 5). Measurements where variation was >10% were discarded and the measurement was repeated.* **(B)** *Particle size distributions at the two scales post low shear application (0.019 × 106 W/kg).* **(C)** *Particle size distributions post high shear application (0.37 × 10^6 W/kg).*

A decrease in live cell population was observed when high levels of shear were applied ($0.37x10^6$ W/kg), along with a concomitant increase in apoptotic and cell debris populations. For the high shear condition, the decrease in the live cell population observed at the 70 L scale was found to be significantly different to that at the 5 L scale (*p* value = 0.002). The apoptotic and the debris populations between the scales were found also to be significantly different (p value < 0.0001). However, these differences in live, apoptotic and cell debris populations between the scales likely indicates a higher level of susceptibility to shear for the material generated at the 70 L scale. The impact or the validity of this difference is not clear as the statistical significance of the data are low, it's therefore recommended to be explored further and to be addressed on a case-by-case basis. .

4.3.3 Generation of mimic feed profiles to primary recovery

In order to create mimic feed profiles with different cell concentrations and viabilities, it was necessary to decouple the control of these factors. Different viabilities were achieved through spiking in material from an independently generated apoptotic cell stock. This section illustrates the capabilities of the proposed method for achieving target conditions. To achieve a reduction in the viability of the material generated using the 5 L STRs, apoptosis was induced in a portion on the cell culture, creating an apoptotic cell stock. A similar method has been shown previously to induce apoptosis early in the culture period (Fransen *et al.,* 2011). A sample of the cell culture material was removed from each culture batch prior to harvest and apoptosis induced in these fractions using cold shock. Apoptosis staining showed a rapid increase in the dead cell population and a decrease in the live cell populations over the course of the following 6 hours (Figure 4.5A).

Overall, a decrease to <20% cell viability was achieved within 7 hours of apoptotic induction. Independent control of cell concentration and viability was demonstrated during the production of the cell culture test material (Figure 4.5B and C). Batches designated "X" and "Y" cell culture material produced in 5 L STRs were harvested on day 14, at viabilities of 65% and 71% and cell concentrations of $8x10^6$, $8.5x10^6$ cells/mL respectively. Post processing, cell concentrations of $100x10^6$ cells/mL were achieved for both batches, while simultaneously achieving different target viabilities of 40% for X and 60% for Y, as shown in Figure 4.5A and B. No significant differences in cell viability beyond that attributable to measurement variability were observed during these cell concentration stages.

The generated apoptotic cell stock was spiked into the concentrated material to achieve the target culture viability levels. Due to the difference in volumetric addition of the apoptotic cell stock derived solution a calculated degree of over-concentration was necessary to achieve the final target cell concentrations for cultures X and Y of $220x10^6$ and $130x10^6$ cells/mL respectively. The degree of over-concentration included the volume compensation for the IgG_1 and the HCP spike volumes. Sample-to-sample variation in both cell concentration and

viability measurements of up to 10% were observed. This was considered to be within the expected measurement variation as dilutions of 50 to 80 times were required to obtain readings within the detection range of the ViCell™.

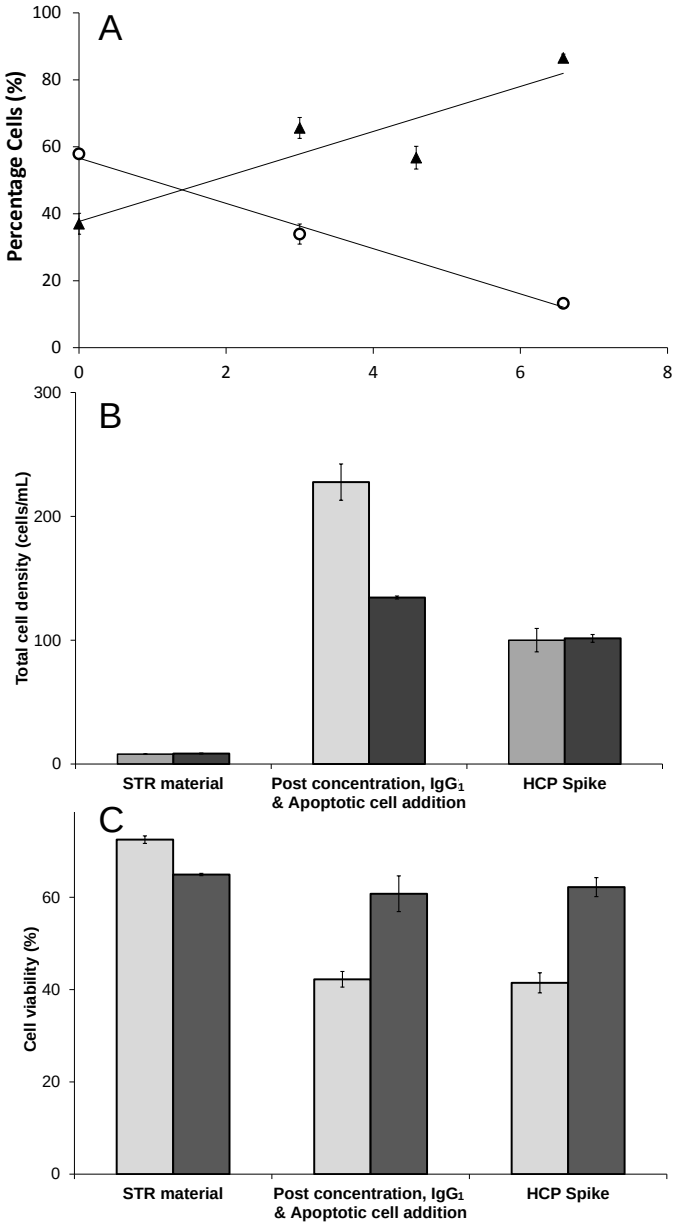

Figure 4.5: (**A**) *Apoptosis induction during the production of a dead cell stock using cold shock is shown. % live (○) and dead (▲) cell populations were measured using FACS staining using the Annexin V-FITC/7ADD kit. Comparison of total cell concentration* (**B**) *and cell viability* (**C**) *achieved in two batches of CCTM generated at the 5-L scale at three key stages of the CCTM methodology. Light grey bars represent Batch X with targeted low viability (40%), and a high cell concentration of 100 × 10^6 cells/mL and a concentration factor of ~28. Dark grey bars represent Batch Y with targeted medium viability (60%), a high cell concentration of 100 × 10^6 cells/mL and a concentration factor of ~15.*

4.3.4 Consistency of mimic feed profiles to primary recovery

The consistency of the CCTM manufacturing methodology was found to vary for each of the target variables. Achieving the target total cell concentration was found to be, within 10% of the target cell concentration. Outliers observed at the higher target cell concentrations of $100x10^6$ cells/mL (Figure 4.6A) were most likely due to the increase in measurement error when using the ViCell™ for high cell concentrations. Viability showed greater variability at low levels of target cell viability. As cells began to break down, the accuracy of the trypan blue method decreased, and the apparent total cell count fell. The percentage viability figure consequently became less consistent and representative (Figure 4.6B). No dead cell stock addition was required for the 70 L SUB material, as the cell viability reduced during the cell concentration stage of the CCTM production. This reduction in viability during the concentration step is consistent with the particle size distribution data (Figure 4.4A, B and C) suggesting that the material produced at the 5 L scale may be less shear sensitive than that produced at the 70 L scale. In addition, the 70 L scale material was produced during the course of a cell culture starting from day 7 to day 14. It has previously shown that day 5-7 cell culture is more susceptible to shear damage and that the susceptibility decreases in the following days (Tait *et al.,* 2009). Apoptotic and non-viable cells have been theorised to be more robust due to the increased porosity of the cell membrane. As a result of this reduction there is a limit to the viability it was possible to achieve post concentration, as the final viabilities were below the target by 15-20%. HCP and titre concentrations were increasingly variable as the target concentrations increased (Figure 4.6C and D). This is also likely to be due to the increase in dilution factors required to quantify effectively the HCP and the IgG_1 concentrations once titre exceeds 10g/ L. The HCP spike consistency was observed to be consistent. The total HCP concentration was kept within 10% batch-to-batch variation.

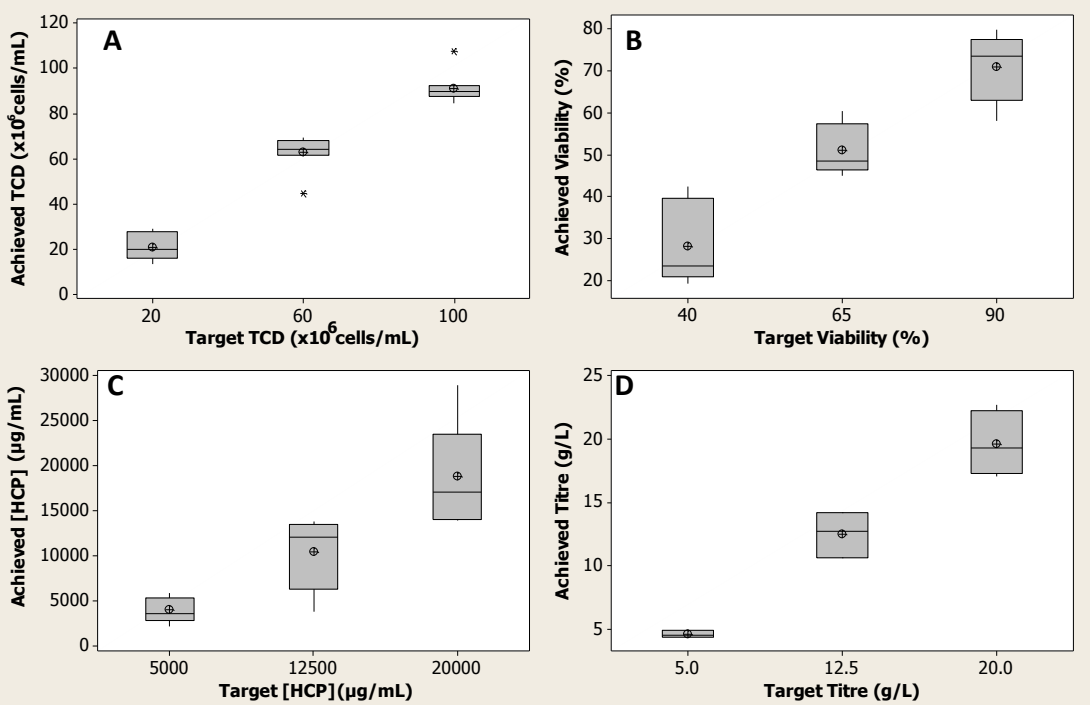

Figure 4.6: *Box plots showing the range in achieved conditions versus the target value for the CCTM. Three target combinations of* (**A**) *cell concentration, (n = 8–10),* (**B**) *viability, (n = 8–10),* (**C**) *HCP concentration (n = 5–8), and* (**D**) *titre (n = 5) were tested. These were manufactured using cell culture material generated at 70L scale. The box represents the 25th hand the 75th percentiles, the median is indicated with a black line, the mean with the star. The whiskers represent data within 1.5 of the interquartile range.*

IgG_1 and HCP profiles were also compared in these three CCTM material batches (Figure 4.7Ai-Ci). IgG_1 and HCPs across the molecular weight and pI spectrum were shown to be present in the HCP stock. Some level of inconsistency in the individual isoform volumes were observed, but the overall diversity in the HCP population between the three batches was consistent. By adoption of these methods it was possible to expose the subsequent processing technologies to a consistent IgG_1 and HCP population within the feed material.

It has been shown previously that towards the end of the culture period mammalian cell culture material typically contains three distinct populations within the particle size distribution: live cell, apoptotic cell and debris populations (Tait *et al.,* 2009). Consistency in particle size distributions of the final CCTM material as well as the HCP population was compared to ensure that the material was as far as possible representative of true cell culture material (Figure 4.7Aii-Cii). In terms of particle size distribution, three populations were observed across the three CCTM batches tested. The large peak at >10µm includes the majority of live cells, the smaller population between 5-10µm in particle size represents the apoptotic cell population and the low volume fraction population with a particle size of <5µm represents cell debris. Particles <3µm were below the detection limit of the instrument and therefore have not been quantified. All three populations were present in the particle

size distributions, and also present in a typical cell culture material originating in an STR. On the basis of this finding it may be calculated that the CCTM methodology provided a consistent and representative feed material input to primary recovery operations in terms of particle size distribution.

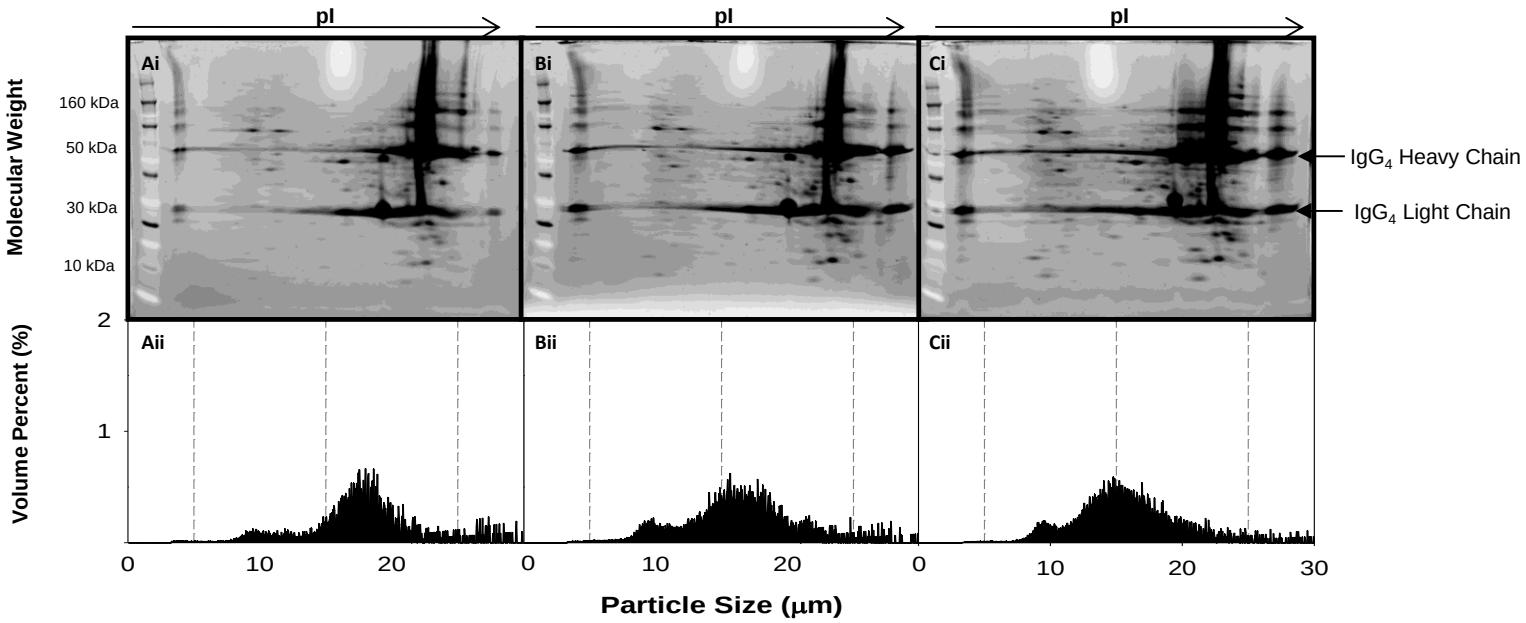

Figure 4.7: *Comparison of CCTM profiles generated using culture material from three 5-L STR cultures in terms of* **(Ai-Ci)**: *HCP profiles, including the IgG1 product and* **(Aii-Cii)**: *equivalent particle size distributions, where % volume fraction of the solids was calculated based on an average measurement of five repeat samples.*

4.4 Conclusions

This chapter set out to develop and demonstrate an experimental methodology for the creation of a mammalian cell culture test material (CCTM). Cell concentration, cell viability, product and impurity concentrations were each manipulated to achieve a range of cell culture test conditions to provide a controlled set of key variables in the cell culture feed to primary recovery. Generation of CCTM material at selected worst case as well as a range of other conditions was shown successfully. Key cell culture parameters effecting primary recovery operation performance including cell concentration, cell viability, product and HCP concentrations were successfully decoupled. An apoptosis induction by cold shock method was applied to create an apoptotic cell population which was used to reduce the viability to selected target conditions. The method was applied to cell culture material originating from bench and pilot scales. Data showed that the ability to target conditions accurately using this method decreased as higher target concentrations were set. It is likely that this feature is, to an extent, an artefact of some of the analytical techniques applied when assaying very high concentrations. The CCTM methodology, created in this work, was able to preserve the expected three distinct particle size populations for live, apoptotic and debris cells with a low batch-to-batch variation. In addition, the selected HCP stock was found to provide a wide variety of HCPs varying in molecular weight and pI, thus providing a basis for determining specific HCP removal capability of a particular unit operation during CCTM application.

Overall, the CCTM method was found to decouple the selected variables in the cell culture feed to downstream operations, while providing consistent material which is representative of typical cell culture material in terms of key features such as particle size distribution and HCP population. The accurate replication of these features make this methodology suitable for application in primary recovery and purification unit operation studies to which they will be applied in the next chapter. Having developed a suitable method to produce test material which matches the criteria identified in Chapter 3, it is now possible to begin to test primary recovery technologies for their potential to cope with high cell concentration, high titre conditions expected in the future.

Chapter 5: Technology Screening for Robust Development of Primary Recovery Strategies for High Cell Density Cultures Using Ultra Scale-Down Models

5.1 Introduction

The two previous chapters focused on defining the likely future demands on primary recovery operations. This was achieved by identifying the current targets for cell culture operations and developing a suitable method to produce test material representative of future cell culture material in terms of high cell concentration, high titre conditions as well as varying impurity loads and viability. These provided the key tools required to begin the development of an experimental framework which would allow the screening of primary recovery technologies in a systematic manner, providing a reliable measure of their practical and economic performance. This chapter describes the screening framework and its application to assess primary recovery technologies for their ability to cope with future cell culture feed profiles.

Primary recovery operations in mammalian cell culture processes have typically been designed to provide high levels of solids removal collectively aiming to remove solids >0.1-0.2 µm in diameter. Centrifugation combined with depth filtration stages have been described as the current workhorses of primary recovery (Liu *et al.*, 2010; Shukla *et al.*, 2007; Yavorsky *et al.*, 2003) in large-scale manufacturing, typically achieving 98-99% solids removal prior to sterile filtration stages. However as cell culture performance has improved dramatically, primary recovery and purification operations have been facing increasingly challenging feed streams, and it is unclear whether current unit operations can continue to provide adequately robust processing options in the future.

Most mammalian cell culture processes used today employ centrifuges fitted with hermetically-sealed feed zones, which reduces the levels of shear exposure. However, energy dissipation levels still reach 0.019 $x10^6$ Wkg^{-1}, which in some cases results in significant cell breakage, and subsequently impacts depth filter area requirements (Boychyn *et al.*, 2004). The key parameter causing variation in solids removal performance at a fixed cell concentration is the cell culture viability at harvest. Generally, efficient solids removal can be achieved at viabilities >50% (Iammarino *et al.,* 2007). For cell culture material with lower viabilities centrifugation is often considered to be more challenging, due to the increased number of cell debris in the processing stream.

At the turn of the century, cell concentrations by mammalian cell culture required relatively low centrifugal discharge frequencies and the contribution to product loss was considered minimal, especially when compared to those experienced in microbial processes (Maybury *et al.*, 2000). Since then, maximum cell concentrations have increased by over 100 fold ($1x10^6$ cells/mL to $>100x10^6$ cells/mL) and average processes now reach 10-20 fold higher

titres (>10 g/L) (Clincke *et al.*, 2011). Subsequently the solids content of the high cell concentration harvest streams is approaching >10% v/v. Centrifuge efficiency at such high solids contents is generally lower and there is a need to desludge the centrifuge more frequently, potentially leading to a greater degree of product loss than previously witnessed (centrifugation unit operation is discussed in more detail in section 1.3.1).

Depth filtration has primarily been used for solids removal of supernatant post centrifugation, and in some cases (generally at scales <2,000 L) to process material directly from cell culture. Most available depth filters use charged filtration media and have been demonstrated to achieve a level of DNA and host cell protein (HCP) removal (Charlton *et al.*, 1999; Yigzaw *et al.*, 2006). Depth filters tend to be followed by absolute pore size rated filters (typically 0.45 µm, 0.2 µm or 0.1 µm) which ensure the removal of solid particulates as well as some endotoxins and a degree of viral removal from the cell culture harvest material (Gerba and Hou, 1985). Together these steps ensure a particle-free product solution, which can then proceed successfully to packed bed chromatography steps.

A wide range of alternative technology options for primary recovery have been previously identified including flocculation (Salt 1995; Barany and Szepesszentgorgyi 2004; Milburn *et al.,* 1990; Habib *et al.*, 2000; Riske *et al.*, 2007), acid precipitation (Lydersen *et al.*, 1994; Westoby *et al.*, 2011), expanded bed absorption (Draeger and Chase 1991; Lutkemeyer *et al* 2001; Poulin *et al.*, 2008), counter current tangential chromatography (Shinkazh *et al.*, 2011) and alternating tangential flow filtration (Schirmer *et al.*, 2010). Although these operations are expected to bring benefits in the future, current limitations in terms of practical application were reported for example potential issues with flocculent presence in the bulk drug substance, low product yields and highly sensitive operational performance (Shukla *et al.*, 2007; Habib *et al.*, 2000). Tangential flow microfiltration (TFF) options, on the other hand, have been suggested in the past to deliver high processing rates without adverse effects on cell viability (van Reis *et al.*, 1982). This advantage can potentially play a key role in reducing cell damage during primary recovery of high cell concentration cell culture feeds, subsequently reducing potential impurity releases. Hollowfibre membranes have primarily been used in mammalian cell perfusion cultures, but are typically not considered for batch type harvest operations. The membrane costs of TFF can be lower than the cost of typical depth filtration media, especially where single use modules are required, as the TFF membrane media tends to be reusable (van Reis 2001).

This chapter focuses on evaluating both current and alternative primary recovery technologies in terms of their robustness and ability to cope with predicted future feed profiles. Cell culture test materials (CCTM) described in the previous chapter (chapter 4), were used for this purpose. The technologies were evaluated based on the following

performance criteria: solids removal, yield and impurity removal. Selected current and alternative technologies were then ranked using a multi-attribute decision-making (MADM) technique and the successful candidates were assessed further using an economic evaluation and facility fit criteria.

The results discussed in Chapter 3 highlighted the increase in demand on solids removal as well as a demand for achieving host cell protein (HCP) and DNA removal prior to purification operations. The results also confirmed a high probability of a change in the feed to primary recovery, where an increase in titre and cell concentration of up to 20 g/L and $100x10^6$ cells/mL respectively were considered likely in the next 12 years. This chapter will focus on firstly identifying some current and alternative technologies with the potential to cope with the future feed to primary recovery, then discuss the results obtained from screening these technologies at the identified worst case conditions, identified by quantifying expert opinion as described in Chapter 3.

5.2 Materials and Methods

A list of technologies which are currently used as well as those which carry probable potential to improve primary recovery operations in the future were identified (see Table 5.1). When choosing the technologies, their potential to achieve impurity reduction was considered as well as their availability for implementation.

Table 5.1: *Current and alternative primary recovery technology systems used in the screening study with corresponding specification description and experimental conditions.*

	System	Specification	Description	Conditions
Current Technologies	Centrifugation	Disc-Stack	CSA-1 mimic *(Westfalia)*	USD set up, mimicking a hermetically sealed feed zone, at a 100 L/h feed flowrate
	Depth filtration	Pre-filter Media (3M)	05 SP: 2-10µm typical particle removal, charged 10 SP: 1-5µm typical particle removal, charged 30 ZA: 1-2µm typical particle removal	USD set up Constant pressure, 100 mbar
Alternative Technologies	Tangential Flow Microfiltration	Bio-Optimal MF-SL™ (Asahi Kasei)	0.4 µm pore size, polysulphone hollowfibre, graduated pore structure	Constant flux, 30LMH
	Tangential Flow Anion Exchange membrane	QyuSpeed D™ (Asahi Kasei)	0.2 µm nominal pore size, anion exchange membrane	Constant flux filtration mode at 30LMH

Table 5.2: *Primary recovery screening study conditions consisting of worst case values for key cell culture variables*

Cell Concentration ($x10^6$cells/mL)	Titre (g/L)	HCP Concentration (µg/mL)	Viability (%)
100	20	20,000	40

Note: The values were determined using results of a survey compiling expert opinion on the likely future cell culture feed profiles to primary recovery. Additionally, a low viability was selected to provide a challenging case for the selected technologies.

The identified technologies were then used for primary recovery of the worst case scenario for potential future cell culture material, i.e. high cell concentration, high titre and high impurity load as well as low viability material (see Table 5.2). Technology performance results were collected and used to carry out an initial technology performance assessment followed by an economic assessment of the technologies. At this stage technologies which met the assessment criteria were carried forward into a design of experiments study to further assess the remaining technologies (see Figure 5.1). This chapter will focus on part 1 of the framework, and part 2 will be discussed in the following chapter.

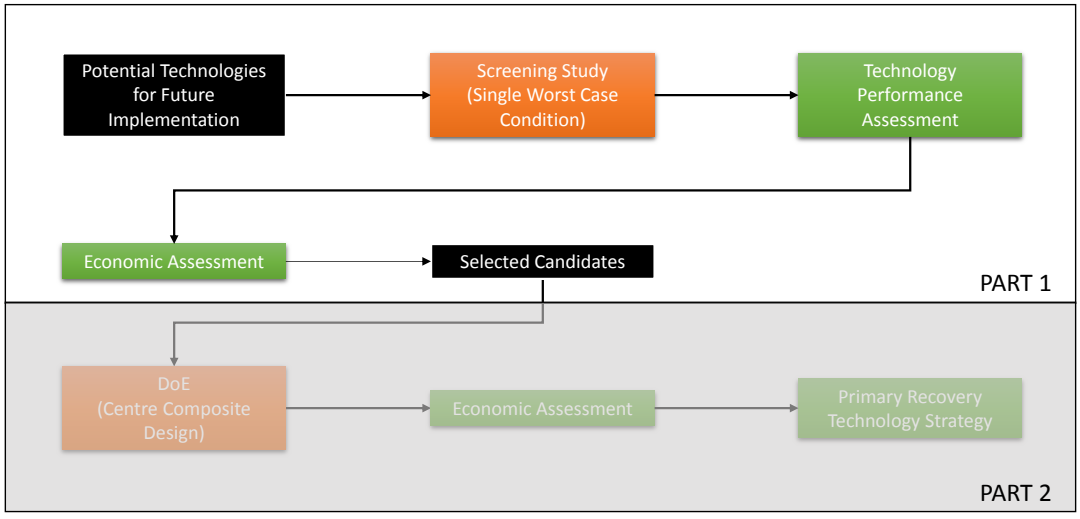

Figure 5.1: *Overview of the integrated experimental and economic framework for primary recovery technology screening.*

5.2.1 Cell Culture and Cell Harvest

Cell culture was carried out in 5L STRs (B. Braun BIOSTAT B-DCU control unit, Sartorius, Epsom, UK) and harvested on day 13. Total cell concentrations at harvest were ~9x10^6

cells/mL with an average viability of 77%. The harvested material was used to generate cell culture test materials with the representative most challenging set of target conditions (Table 5.2). The CCTM generation is discussed in detail in the previous chapter. The cell culture harvest was concentrated and spiked with the volumes of IgG_1 and a HCP stock to create the required conditions. Apoptosis induced cell stock was added to the CCTM in order to achieve the target viability of 40%.

5.2.2 Primary Recovery Methods

Technologies were selected to represent current and alternative primary recovery options (Table 5.1). Three centrifugation (hemetically-sealed disc-stack centrifuge) and primary depth filtration options (05SP, 10SP and 30ZA media options) were selected to represent the current options, and two tangential flow filtration options (Bio-Optimal MF-SL™ and QyuSpeedD™) to represent the alternative options. The CCTM were then used as feed to these unit operations. The methodology and the running conditions applied in each case are described in section 2.3.

5.2.3 Technology Assessment

A weighted sum multi-attribute decision making (MADM) technique (DeGarmo *et al.*, 1988; Farid *et al.*, 2005; George *et al.*, 2007; Pollock *et al.*, 2013) was used to combine the performance data into a single metric. Initially, the calculated technology performance values for each attribute were normalised to a 0-1 scale, where the zero value represented the worst case performance result and value of one represented the best case performance result.

$$N = \frac{P_A - P_{\min}}{P_{\max} - P_{\min}} \tag{5.1}$$

where P_A is the actual figure for a performance attribute (e.g., solids removal), P_{min} is the minimum value achieved by the technologies and P_{max} is the maximum value achieved by the technologies for the same performance attribute. The normalised values were then weighted using a ratio of 3:2:1 for the performance attributes of solids removal to DNA removal to HCP removal. This ratio was selected based on a survey carried out to quantify industry opinion on demands facing primary recovery operations in the future (see section 3.3). The subsequent sum of the weighted values for each technology leads to an overall rank figure (*OR*) from which technology performances can be compared.

$$OR = \sum_{i=1}^{3} \omega_i N_i \tag{5.2}$$

where ω_i is the weighted value and the N_i is the normalised value each corresponding to the performance of metric *i*. The resultant *OR* also has a value between zero and one, therefore representing the least and the most efficient option for a given scenario respectively. Selection criteria of the current typical minimum performance in terms of solids removal, yield and impurity removal were used as cut off criteria for technology performance.

5.2.4 Economic Evaluation

The economic evaluation was focused on the primary recovery stages only using the same worst case input conditions as were generated in the practical experiments (Table 5.1, Table 5.2). These were combined with the sizing data collected during the experimentation and further assumptions (Table 3 and 4) to calculate equipment duties required, kg product outputs per batch, capital investment and cost of goods (COG_{PR}) outputs for the primary recovery operations for three scale scenarios: 2,000 L, 10,000 L and 20,000 L production scales. A detailed process economics model was built in Excel (version 2010) that integrated mass balance, design and cost equations so as to generate the key performance metrics for the different primary recovery strategies. The mass balance and design equations accounted for features such as the impact of the cell concentration on the number of centrifugation discharges required (calculated based on the centrifuge model used to obtain experimental data) and the resulting yield loss. Experimental results were used as inputs for worst case filter throughputs and flux likely to be achieved. The cost equations were similar to those detailed in Farid *et al.,* 2007.

5.2.5 Economic Evaluation Assumptions

Primary recovery operations were defined as those activities involved in the processing of cell culture material during cell culture harvest until the completion of the sterile filtration stage prior to chromatographic purification. Sizing data including throughput, yield and solids removal were collected using the experimental set up described in Table 5.2. This was combined with additional scenario constraints to provide the final sizing outputs. The scenario constraints included a target processing time of 6 hours. In addition, details on equipment performance at the selected scale were constricted to specific equipment choices. This included a choice of Alpha Laval BTAX215H and Alpha Laval BTAX205 centrifuges, lenticular mobile skids for depth filtration, 8 m^2 and 5 m^2 modules for the Bio-Optimal MF SL™ and the QSD™ options respectively.

Production was assumed to consist of 17 batches per year, with a process length assumed to be 20 days. The COG_{PR} comprised of both the direct and indirect costs for primary recovery operations only, assuming use of an existing facility. The direct costs included materials (e.g. filters, single use materials etc.), labour costs (including operational labour)

and WFI costs. The indirect costs included depreciation of 10% per annum over 10 years based on the capital investment. Capital investment was calculated on reusable equipment (e.g., filtration skids, centrifuge units etc.) and auxiliary equipment (e.g., pumps) using Lang factors shown in Table 5.3, Table 5.4 and Table 5.5.

Table 5.3: *Key assumptions and calculations used to construct the process economics model.*

Unit Operation Inputs	Source	Outputs	Calculations
Centrifugation			
Cell culture volume, V_T	Scenario input		
Wet mass per cell, M_{WC}	Experimentally determined	Cell mass in the feed	$TCD \times V_T \times M_{WC}$
% of solids fraction remaining, S_R	Experimentally determined using particle size distribution	Solids mass in the sediment S_T	$(100\text{-}S_R)/100$
Centrifuge models available: - Alpha Laval BTPX-205 - Alpha Laval BTAX215H	Scenario input		
Solids holding capacity, S_H			
Dewatering level – 50%	Manufacturer's specification	Mass of liquor in the sediment, M_{LW}	$0.5(S_H)$
Number of centrifuge units, U	Facility specification, = 1 specified unit	Number of discharge operations, N_P	$(S_T/S_H)/N_U$
Maximum feed flowrate, Q_{Cin}	Manufacturer's specification	Total liquor loss in the sediment V_W	$((N= \times V_W)\text{-}(S_T/\rho_S)) \times U$
Product is only lost in the liquor fraction of the sediment	Assumption	Processing time, t_{PCent}	$V_T/(UxQ_{Cin})$
Depth Filtration			
Product mass in, m_{Pin}	Input from centrifugation calculations	Volume in, V_{DFin}	$V_T - V_W$
Throughput V_{max}	Experimentally determined		
Safety factor S_F	Assumption = 1.5	Total membrane area required, A_{DF}	$(V_{DFin}/V_{max}) \times S_F$
Membrane area per module, A_M	Manufacturer's specification	Number of modules required, N_m	A_{DF}/A_M

Number of modules per skid, N_{MSK}	Manufacturer's specification	Number of skids required, N_{SK}	N_m/N_{MSK}
Hold up volume per module V_{DFhup}	Manufacturer's specification	Permeate volume, V_{DFp}	V_{DFin}-(V_{DFhup} x N_m)
Unit specific product concentration yield, Y_{CP}	Experimentally determined	Product Yield, Y_{DF}	m_{Pin} /(m_{Pin}/ V_{DFin} x (Y_{CP}/100) x V_{DFp})
Skid size: length and width, *l,w*	Manufacturer's specification		
Minimum space required for operation, OP_A	Assumption = 1m	Floor space required, A_{FSP}	((l+ OP_A)x(w+ OP_A)) x N_{SK}

TFF

Cell culture volume, V_T	Scenario input		
Throughput, *T*	Experimentally determined		
Safety Factor, S_F	Assumption = 1.5	Total membrane area required, A_{TFF}	(V_T/*T*) x S_F
Membrane area per module, A_M	Manufacturer's specification	Number of modules required, N_m	A_{TFF}/A_M
Number of modules per skid, N_{MSK}	Manufacturer's specification	Number of skids required, N_{SK}	N_m/N_{MSK}
Hold up volume per module V_{TFFhup}	Manufacturer's specification	Permeate volume, V_{TFFp}	(A_{TFF}/A_M) x V_{TFFhup}
Unit specific product concentration yield, Y_{CP}	Experimentally determined	Product Yield, Y_{TFF}	m_{Pin} /(m_{Pin}/ V_{DFin} x (Y_{CP}/100) x V_{TFF})
Skid size: length and width, *l,w*	Manufacturer's specification		
Minimum space required for operation, OP_A	Assumption = 1m	Floor space required A_{FSP}	((l+ OP_A)x(w+ OP_A)) x N_{SK}

Cost of Goods Model Breakdown

Cost Category	Value

Direct Cost	Materials:	
	Filter Modules	*f(utilisation)*
	Sterile Filters	
	Tubing	
	Miscellaneous Materials	
	Labour	
	Operating Labour	
	WFI	
Indirect Cost	Maintenance	*0.1xCapital investment x Project duration*
	Depreciation	*Capital investment/Depreciation period x Project duration*
	General Utilities (HVAC)	*Cost per unit area x Facility size x Project duration*

Table 5.4: *Key cost assumptions used in the construction of the process economic model.*

Resource	Specification	£/Unit	Unit
Equipment			
Stainless steel vessel	2,000 L	65,700	£/unit
	10,000 L	71,500	
	20,000 L	174,200	
Peristaltic pump	2,000 L/h	2,750	£/unit
Alpha Laval BTX-205 centrifuge	2,500L/h	150,000	£/unit
Alpha Laval BTAX 215H centrifuge	12,000L/h	577,420	£/unit
Depth Filtration Rig	Standalone Rig System	20,000	£/unit
TFF Rig	Mobile skid	30,000	£/unit
Labour			
Operating labour	Single operator with maximum shift length of 8h	300	£/h
Materials			
Depth Filtration 30ZA modules	3.2m^2 per module	400	£/module
TFF: Bio-Optimal MF-SL modules	8m^2 per module	9,847	£/module
TFF: QSD modules	5m^2 per module	20,520	
WFI	Generated in-house assumed	100	£/L
Tubing		10	£/m
Sterile filter	0.22 µm	200	£/m^2

Table 5.5: *Bioprocess facility capital investment factors and corresponding Lang factor included in the indirect cost calculations.*

Lang Factor Description	Cent&30ZA Base Case	TFF Reusable Technology
Total Equipment Purchase Cost (inc. utilities)	-	1
Pipework & installation	-	0.9
Process control	-	0.37
Instrumentation	-	0.6
Commissioning	-	0.07
Equipment Validation	-	0.06
Contingency factor	-	1.15
Lang factor	**1.00**	**3.45**

5.3 Results and Discussion

5.3.1 Primary Recovery Technology Performance

The performance of three centrifugation and depth filtration options (05SP, 10SP and 30ZA media) as well as two tangential flow filtration unit options (Bio-Optimal MF-SL™ and QSD™) were investigated when challenged with the most demanding cell culture material conditions (see Figure 5.2). A USD set up was used to mimic the characteristics of a disk-stack centrifuge operating with a hermetically-sealed feed zone and a feed flowrate of 100 L/h. The centrifuged material was then passed onto the USD depth filtration set up using a 0.28 cm^2 depth filter disc of each of the three media types at a constant pressure of 100 mbar. The resultant filtrate was compared to the material which passed through the single fibre set up of the Bio-Optimal MF-SL™ module (4 cm^2) and the QSD™ (6 cm^2) at 30 LMH constant flux. Comparisons were made in terms of solids removal, yield and levels of impurity removal achieved.

Initially solids removal was measured using OD measurements and subsequent calculation of the % solids removal, but this proved unreliable due to the high levels of fine particles generated during the USD centrifugation step. Therefore solids removal for the USD centrifugation step was further analysed by dry cell weight measurement of the supernatant compared to the feed and was also normalised using the supernatant passed through a 0.22 µm filter. This presented a more reliable measure of the centrifugation performance. Solids removal was measured for the depth filtration options using the OD measurement methodology and was used as a rough filter performance indicator. Direct comparison

between centrifugation and depth filtration solids removal capacities was not made due to the differences in the quantification techniques applied.

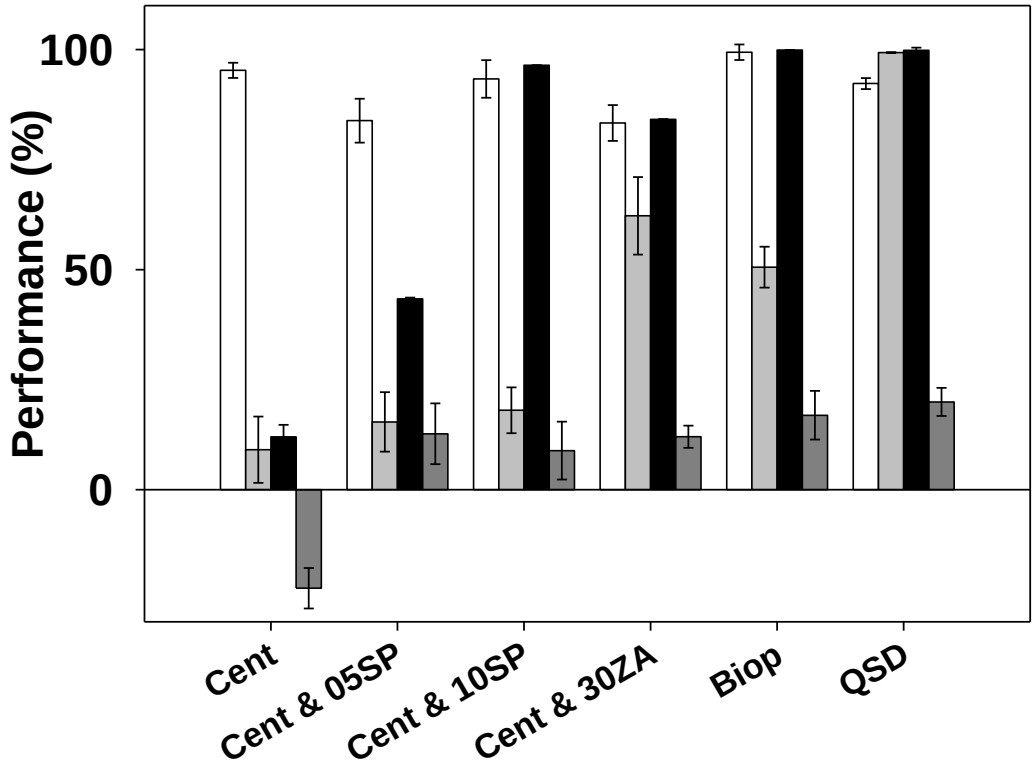

Figure 5.2: *Primary recovery technology performance under the worst case screening study conditions generated using the CCTM in terms of % yield performance (□), %DNA removal performance (□), %solids removal (■) and % HCP removal (■). The worst case screening study conditions are summarised in Table 5.1.*

HCP release into the supernatant stream was also observed, indicating high levels of cell damage due to shear. The particle size distribution data (see Figure 4.4**Error! Reference source not found.**) of the feed material and the supernatant also showed an increase in particles between 2-5 µm in diameter, and the presence of some particles between 5-10 µm in diameter. An absence of particles with diameters above 10 µm in the supernatant stream, was observed. The 30ZA option however provided the highest degree of solids removal compared to the 5SP and 10SP options, as it has the smallest nominal pore size. However, breakthrough occurred during filtration and this increased the sample pool OD. A ~10% total HCP removal was observed when using all three media types, as well as substantial DNA removal of 15-20% and 63% DNA removal using the SP and the ZA media respectively. The observed difference in removal was expected and can be explained by the positive charge provided by the ZA media compared to the SP. The low HCP removal observed using the depth filtration medium is within the current reported literature range for a single medium containing filtration device (Yigzaw *et al.,* 2006). The DNA removal using the ZA medium

also falls within the expected range reported in literature (Dorsey *et al.,* 1997, Charlton *et al.,* 1999).

The Bio-Optimal MF-SL™ and the QSD™ options achieved >99% solids removal, however their performance differed in terms of impurity removal levels. Operating with the Bio-Optimal MF-SL™ provided a ~50% DNA removal compared to >99% DNA removal using the QSD™ and HCP removal of 15 and 20% respectively. The Bio-Optimal MF-SL™ media is however uncharged and therefore was not expected to result in a high level of DNA removal. However high cell mass within the hollowfibre may have resulted in entrapment of DNA due to a combination of physical particle retention and non-specific binding to the cellular matter. The QSD™ was expected to achieve a high level of DNA removal due to the high charge capacity provided by the diethylamine (DEA, NH((CH2CH3)2) ligands grafted to the polyethylene (PE) membrane material (Shirataki *et al.*, 2011). A 99% removal of DNA has not been previously reported using centrifugation and depth filtration combinations. However, specific benefits of DNA removal at the earlier stages of the process are not clearly communicated and would benefit from quantification as part of future work.

In terms of particle size distribution, the Bio-Optimal MF-SL™ showed the lowest presence of solids across the particle size range. The QSD, BioOptimal as well as the centrifugation and 30ZA options showed an overall low solids content, with the solids fraction remaining below 3%.

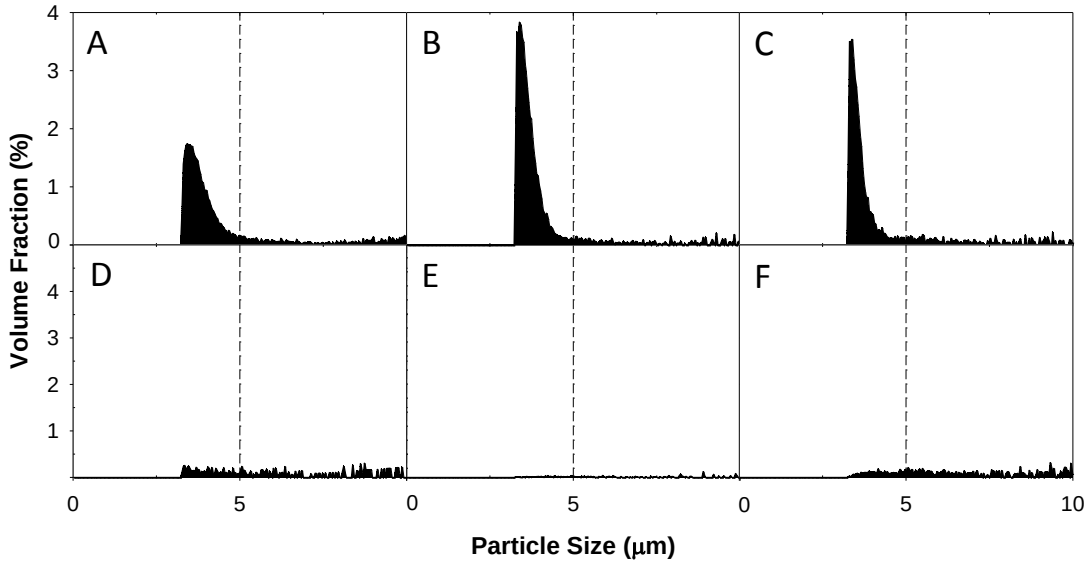

Figure 5.3: *Particle size distributions of samples post solids removal using the different primary recovery technology options. (A) supernatant fraction post USD centrifugation, (B) permeate fraction post centrifugation followed by depth filtration using 05SP media, (C) permeate fraction post centrifugation followed by depth filtration using 10SP media, (D) permeate fraction post centrifugation followed by depth filtration using 30ZA media, (E) permeate fraction post tangential flow filtration using Bio-Optimal MF-SL™, (F) permeate fraction using QSD™ in tangential flow filtration mode.*

5.3.2 HCP Removal Profile

One of the key functions of the unit operations following the solids removal/clarification stages is HCP removal. Protein A typically removes ~95-99% of HCPs (Ghose *et al.*, 2005). However, HCP reduction prior to Protein A may have benefits in terms of Protein A resin lifetime. In addition, specific HCP removal may still be a concern for some processes or cases where upstream batch-to-batch variability may cause expression of HCPs which are particularly difficult to remove. Therefore, the HCP removal potential of each technology option was investigated further in order to gain an understanding of the types of HCPs these technologies tend to remove. 2D PAGE gels of the starting material and post primary recovery operations were run in triplicate. HCP normalised spot volumes were compared to the CCTM gels in four gel areas numbered Q1-Q4 (Figure 5.4A), representing different pI and molecular weight combinations (low pI and low molecular weight; high pI and low molecular weight; low pI and high molecular weight; high pI high molecular weight). Spot increases and decreases were calculated relative to the CCTM gels and included any new

spots which were not originally found on the CCTM gel images (Figure 5.4B). This method was used to indicate any specific areas of HCP removal which a particular technology can provide. Highest spot number decreases were seen in the low molecular weight and high pI region across all the technologies (Q4). Centrifugation & 30ZA, Bio-Optimal MF SL™ and QSD™ options also showed higher HCP removal in the Q3 region. Highest spot increases were observed in the Q1 region for all the technologies except the centrifugation & 30ZA option. The centrifugation and 30ZA option showed higher net spot decreases than increases across all the quadrants.

A dark line of unresolved lower pI HCPs can be seen on the feed material gel (Figure 5.4A). Due to the poor resolution these were not quantified, but a significant reduction of this region was observed post QSD™ application and some reduction was observed post Bio-Optimal MF-SL™ application (see images in Appendix C).

Changes in HCP spot volumes during primary recovery operations have been documented previously (Tarrant *et al.,* 2012; Tarrant *et al.,* 2014). Work was also carried out to identify the HCPs which showed a significant change in volume between the operations (Hogwood *et al.,* 2015). Although such identification shows the relevance of the findings reported in this thesis, it also highlights that the presence of specific HCPs is highly cell line dependent. Therefore, this type of comparison is suitable as part of a method applied for technology selection and is recommended to be carried out with the cell line of interest.

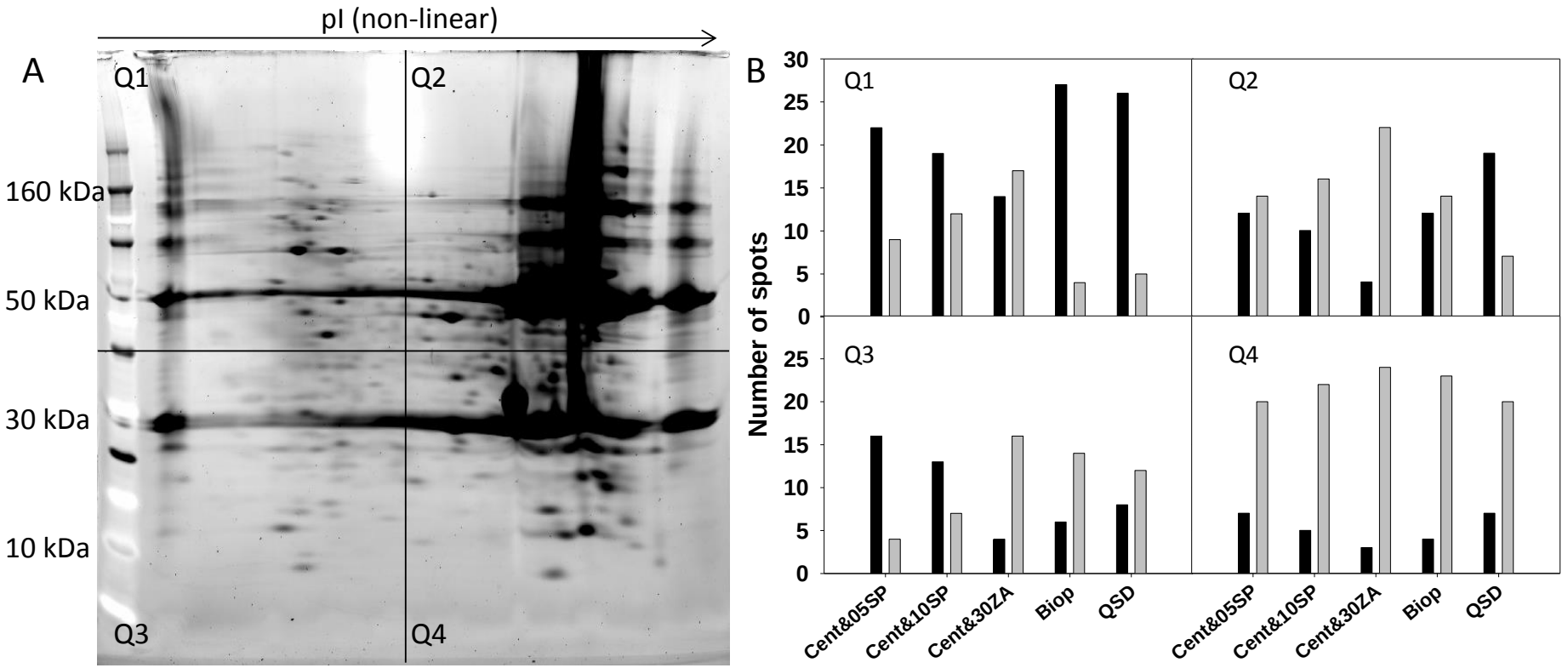

Figure 5.4: *2D PAGE gel analysis of CCTM feed and samples post primary recovery using each of the selected technologies. The gels were divided into 4 quadrants based on gel coordinates. A: 2DPAGE gel of the CCTM feed divided into four quadrants - (Q1) low pI and low molecular weight region, (Q2) high pI and low molecular weight region, (Q3) low pI and high molecular weight region, (Q4) high pI and high molecular weight region. B: Increases (■) and decreases (■) in the normalised spot volumes compared to the CCTM feed in each quadrant using SameSpots software.*

5.3.3 Technology Assessment

An MADM additive weighting technique was used to assess the practical findings and quantify the overall performance by considering the performance attributes taken together. A potential scenario for the selection criteria was then explored, where yield and purity targets were selected based on the current platform processes as well as literature data.

Solids removal, DNA reduction and HCP reduction results were normalised based on the minimum and maximum values for each attribute to obtain a rating value between zero and one (Table 5.6). The example values for a current platform are also shown. The ratings were then obtained for each technology and each of the selected performance attributes. An overall purity weighted score was then obtained and compared across the technologies (
Table 5.7). These results reflected the practical data, where the QSD™ displayed the highest score, followed by Bio-Optimal MF-SL™, the centrifugation plus 30ZA, centrifugation plus 10SP and finally centrifugation & 05SP option. Based on the normalised weighted score of the "current operational level" selected, centrifugation & 05SP as well as centrifugation & 10SP options were seen to fall below the desired level, while centrifugation & 30ZA, Bio-Optimal MF-SL™ and the QSD™ options performed above the set base line. Additional minimum yield criteria was then also implemented and set to be 90% (Figure 5.5). As a result the Bio-Optimal and the QSD™ technologies met both of the set criteria while the centrifugation and 30ZA option met the target purity criteria but not the yield criteria. Centrifugation and the 10SP option met the yield criteria but not the purity criteria and the centrifugation and the 05SP option met neither of the criteria. If reducing the acceptable yield criteria to 80% is possible centrifugation and 30ZA option would not be ruled out. Accounting for this possibility we did not rule out the centrifugation plus 30ZA option at this stage.

Table 5.6: *Values representing typical existing operational performance for primary recovery technologies. These were combined with the projected technology performance results to obtain normalised technology performance ratings using minimum and maximum values in respective performance categories.*

Performance attribute	Current operational performance	Min value (%)	Max value (%)
Solids removal (%)	98	43	100
DNA Removal (%)	30	15	62
HCP Removal (%)	10	9	17

A sensitivity analysis was performed on the weighting ratio of DNA:HCP removal to determine the impact on the ranking of the technologies. Figure 5.5 illustrates that if the HCP removal score is considered more important than DNA removal (DNA:HCP removal weighting ratio < 0.75), the normalised weighted score of the centrifugation plus 30ZA option falls below the acceptable threshold values. The ranking of centrifugation plus 05SP and centrifugation plus 10SP options also switches at a DNA:HCP removal ratio below 0.25 where centrifugation plus 10SP option scores fall below the

05SP alternative. These trends are driven by the very small differences in HCP removal performances of the technologies and the much greater differences in DNA removal performances.

Table 5.7: *Normalised performance ratings (0-1) for each primary recovery technology option.*

Primary recovery options	Solids removal rating	DNA removal rating	HCP Removal Rating	Overall Weighted Score	Normalised Weighted Score
Current Operation Level	1.0	0.0	0.1	3.1	0.5
Centrifugation plus 05SP	0.0	0.2	0.3	0.7	0.1
Centrifugation plus 10SP	0.0	1.0	0.0	2.0	0.3
Centrifugation plus 30ZA	0.6	0.8	0.3	3.5	0.6
Bio- Optimal MF-SL™	0.4	1.0	0.7	4.0	0.7
QSD™	1.0	1.0	1.0	6.0	1.0

Note: The normalised values were calculated, using minimum and maximum performance data obtained experimentally. Performance categories were weighted 3:2:1 (solids removal: DNA removal: HCP removal) in order to obtain a normalised weighted score for each technology.

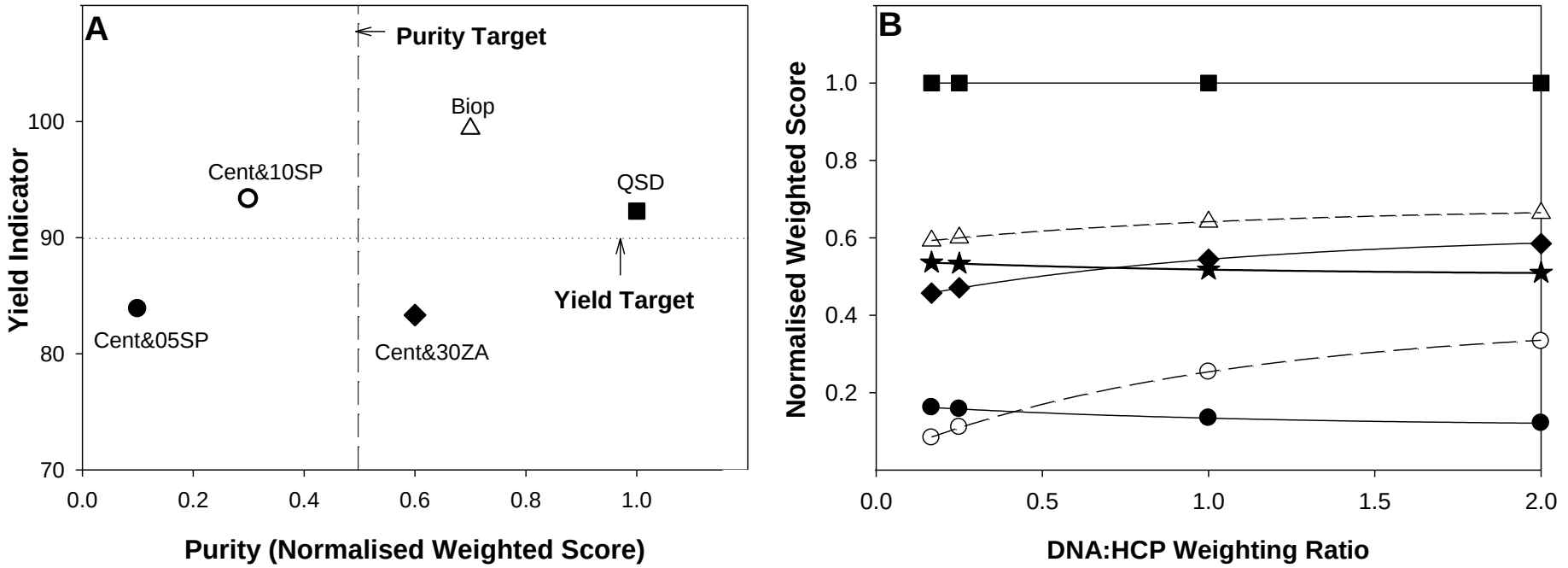

Figure 5.5: *Primary recovery technology performance scores calculated using an MADM additive weighting technique. The normalised weighted score was calculated for purity by assuming a 3:2:1 weighting ratio of solids removal : DNA removal : HCP removal. These scores are presented for centrifugation and 05SP depth filtration media option (●), centrifugation and 10SP depth filtration media option (○), centrifugation and 30ZA depth filtration media option (◆), Bio-Optimal-MF-SL™ option (△), and the QSD™ option (■). The scores were plotted against the yield result obtained in terms of product concentration. Technology performance targets were applied based on existing processes to obtain a yield target and a purity target for technology selection. B: Sensitivity analysis on score weighting was performed by altering the DNA : HCP weighting ratio. Normalised weighted score for the selection criteria rating based on current operational performance.*

5.3.4 Economic Evaluation

In order to compare the economic impact of the primary recovery technology selection, the cost of goods was calculated for the primary recovery operations defined as all activities post cell culture harvest, not including Protein A purification operation and beyond.

Throughput data for the three depth filtration options and the Bio-Optimal MF-SL™ and the QSD™ was used in the comparison. The Bio-Optimal MF-SL™ was found to provide the highest capacity of > 110 L/m^2, followed by the 05SP, 10SP QSD™ and 30ZA media (101, 88, 82, 32 L/m^2 respectively). This is consistent with a previously reported throughput range seen in the SP filtration media when processing high cell concentration material (Pegel *et al.*, 2011).

This data together with the unit operation assumptions and the performance data presented in the previous section provided the inputs to the cost of goods model (Table 5.1). Based on the technology performance results, economic outcomes are presented for those technology options which fulfilled one or more performance target criteria (i.e. centrifugation and 30ZA; Bio-Optimal MF-SL™; QSD™). The centrifugation and 30ZA media option achieved the lowest kg/batch output of product, when compared to both of the TFF options (see Figure 5.6A). Based on model evaluation, this low output was found to be due to the yield losses expected during the large scale centrifugation operation. At such high solids concentrations, the number of discharge operations required by the selected centrifuge models, reduced the overall step yield from ~90% to 30-40% at the three scale scenarios. This had a considerable impact on the cost of goods (RMU/g) output (Figure 5.6B), across the scales where the centrifugation and 30ZA option is the most costly followed by the QSD™ and the Bio-Optimal MF-SL™. The RMU/g figures were benchmarked against a commercially available software, which yielded results within the same order of magnitude.

The space required to facilitate the use of the equipment required was also investigated (Figure 5.6C). The centrifugation & 30ZA media was found to require > 200 m^2 of floor space to accommodate the skids at the 20,000 L scale, which totalled 23 units. A current 20,000 L facility was used as a benchmark to provide facility fit constraints. It can accommodate 10 skids for primary clarification filters and 10 skids for secondary clarification filters (as the current worst case scenario). The space required to accommodate the 30ZA primary clarification skids at the 20,000 L scale at the set scenario conditions was more than double this. Although the number of skids required to accommodate the QSD™ option was also high, secondary filtration prior to the sterile filters is not required, and would fit into this hypothetical facility. The Bio-Optimal MF-SL™ option required approximately a quarter of this floor space. This indicates that in cases where existing facilities are used for new processes or alternative unit operations, the TFF options may prove to be more easily accommodated.

Material costs dominate the COG_{PR}/batch in all technology cases across the scales (Figure 5.7A). The QSD™ option had the highest cost per batch, which is approximately double the cost of the centrifugation and 30ZA option and over three times the cost of Bio-Optimal MF-SL™. This is due to the high cost per module, as the technology is designed and priced to compete with anion exchange resins and membranes (Table 5.4). The QSD™ module cost comprises 80% of the total COG_{PR}/batch compared to the 60-70% in the centrifugation and 30ZA and Bio-Optimal MF-SLTM cases (Figure 5.7B). In addition, WFI costs make up 10-20% of the batch cost in the case of the TFF options compared to 2% in the case of centrifugation and 30ZA.

The lowest cost operation across the scale scenarios is the Bio-Optimal MF-SL™, due to the high throughput it achieves and the relatively low cost of the modules compared to the QSD™. Although increasing membrane reusability beyond 50 times significantly reduces the RMU/g output, a reusability of 10 times was selected for these scenarios to represent a suitable option for a CMO. However, options including centrifugation can benefit from economies of scale once a larger centrifuge model is required (scales >5,000L).

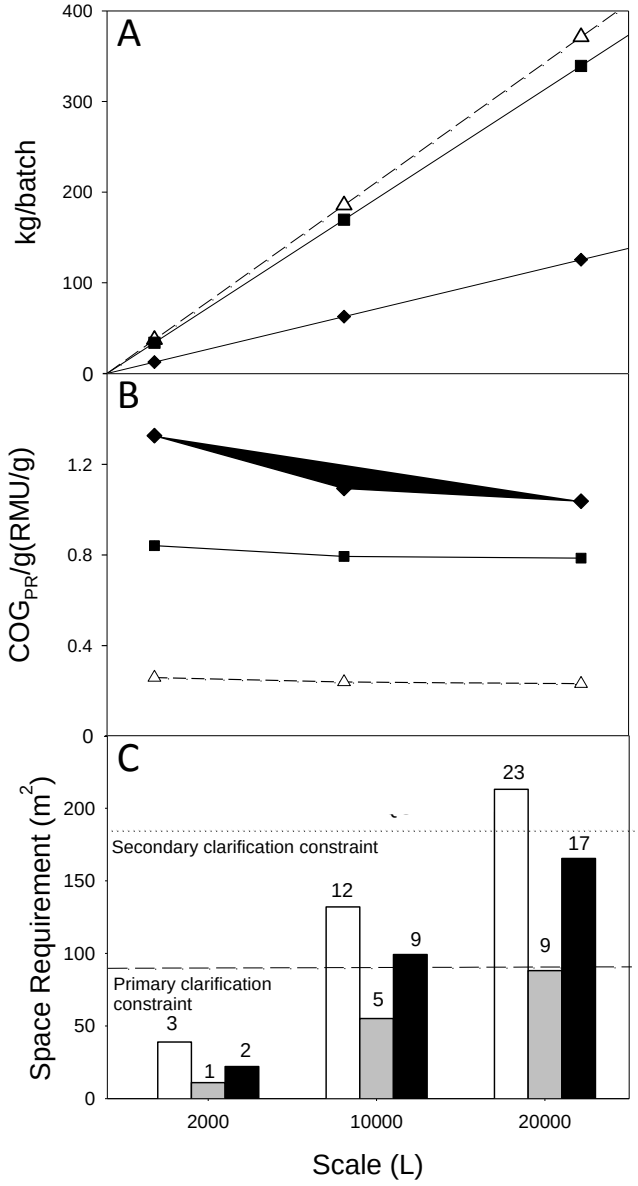

Figure 5.6: *Kilogram per batch output (A), COG_{PR}/g (B) and floor space required (C) for the primary recovery technology options at three scale scenarios of 2,000 L, 10,000 L and 20,000 L. Analysis based on experimental performance at the selected worst case feed to primary recovery conditions, assuming 17 batches per year production at each scale. Cost of goods (COG_{PR}/g) account for primary recovery costs only. Floor space considerations assumed a minimum of 1m operational space around each unit. Results are presented for the centrifugation & 30ZA option (◆; □), Bio-Optimal MF-SL™ option (△; ▨),; QSD™ option (■;■). In C, the numbers above the bars indicate the number of filtration skids required in each given scenario. An example of an existing current worst case primary recovery space requirement for a 20,000L process is indicated for primary clarification (– – -) and primary and secondary clarification collectively (·····).*

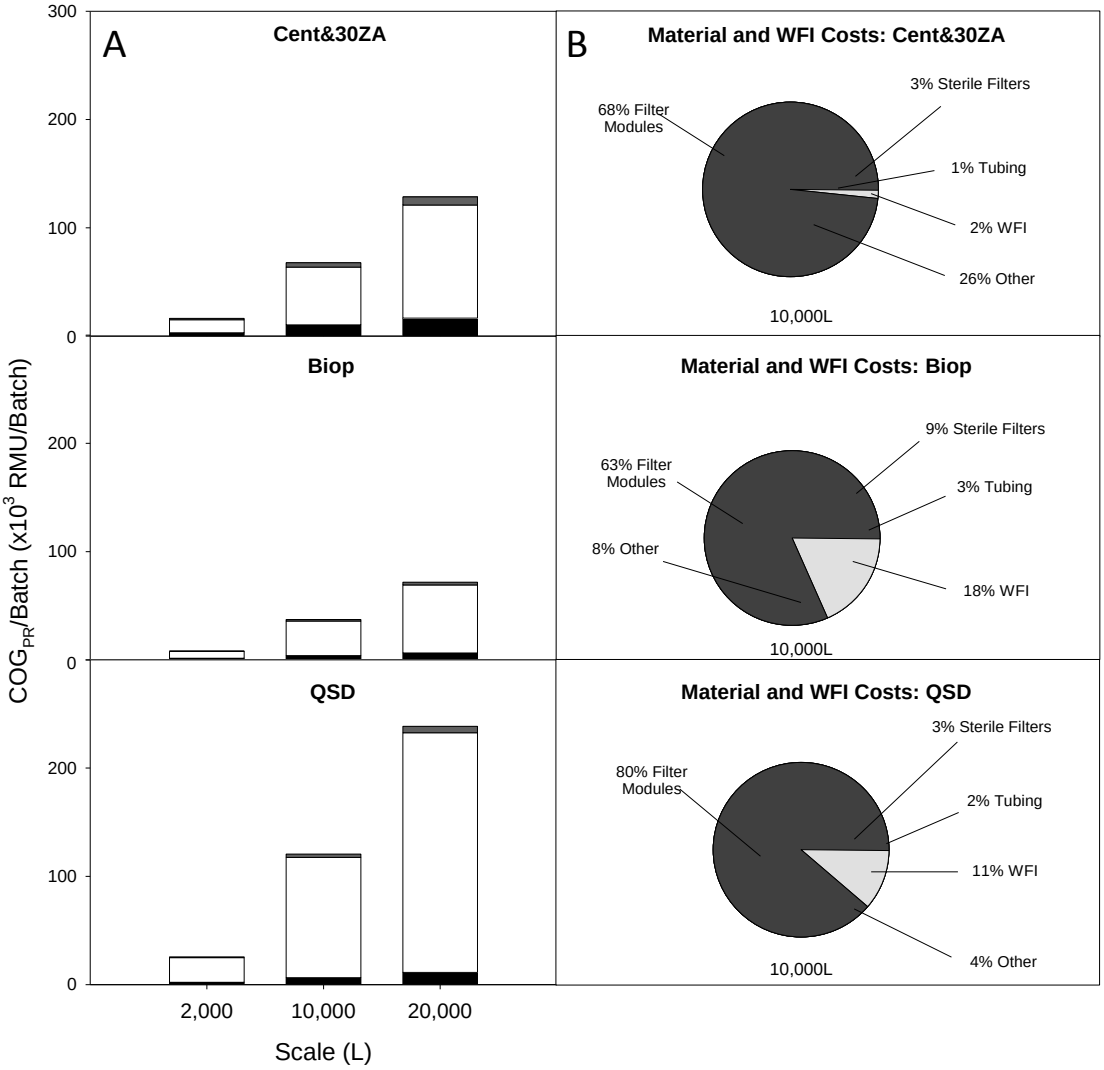

Figure 5.7: *A: Comparison of COG_{PR} breakdown at each scale scenario on a category basis for indirect costs (■), material costs (□) and labour costs (▩), for the primary recovery operations only. B: Material and WFI costs breakdown at the10,000 L scale scenario.*

5.4 Conclusions

Using a combination of cell culture test materials (CCTM), ultra scale-down technologies, multi-attribute decision-making methods, process economics and facility fit considerations, this chapter has demonstrated a methodology and results for achieving a screening of current and alternative primary recovery technologies. The example technologies tested using this method included three centrifugation and depth filtration options (using 05SP, 10SP and 30ZA media), and two alternative tangential flow filtration options (using Bio-Optimal MF-SL™ and QSD™ hollowfibre modules). HCP removal levels in the range of 10-20% were reached across all the tested technologies, however removal of specific HCP groups varied. Up to 99% DNA removal was achieved using the QSD™, with lower levels of DNA removal using the other options.

MADM analysis as well as selection based on current technology performance criteria showed that only two options met the yield and purity criteria: Bio-Optimal MF-SL™ and the QSD™. The centrifugation and the 30ZA option met the purity criteria but not the yield criteria. The options were further evaluated based on their economic performance. This showed the centrifugation and 30ZA option to be the least cost-effective across the 2,000 L, 10,000 L and 20,000 L scale scenarios and not fit the facility constraints set based on a typical existing large-scale facility. The Bio-Optimal MF-SL™ option was the most cost-effective option across the 2,000-20,000 L scales of operation.
Economic and MADM analysis of the alternative technologies has been used to predict robust primary recovery options for the future. The QSD™ was found to provide greater capability for DNA removal prior to purification operations, however remained a costly alternative. The Bio-Optimal MF-SL™ offered a similar level of solids removal but was more cost-effective.

These findings provided a single extreme data point, initially defining and assessing the potential of the technologies to cope with worst case scenarios. The outcome prioritised the two alternative technologies to investigate further in the next study, which focused on further exploring their performance over a range of cell culture conditions between the current and the future worst case condition. The next chapter outlines part 2 of the framework, which focuses on mapping Bio-Optimal MF-SL™ and QSD™ performance as a function of key cell culture variables.

Chapter 6: Effects of key variables in mammalian cell culture broth on primary recovery by tangential flow microfiltration

6.1 Introduction

The previous chapter identified two alternative technologies Bio-Optimal MD-SL™ and QSD™ (both tangential flow filtration options) with the potential to provide robust primary recovery options for the future. Both options fulfilled performance and economic screening selection criteria under the worst case scenario. However, current cell culture conditions are 10-20 fold lower than the worst case conditions tested and therefore may not require a switch to the identified technologies for the moment. To understand the point at which a technology change to either of the alternatives becomes viable, this chapter focuses on mapping the performance of the alternatives as a function of the cell culture variables.

Cell concentration and cell viability have been previously shown to impact tangential flow filtration performance in terms of throughput prior to solids breakthrough. The need for robust performance at a range of cell concentrations and viabilities has been identified in Chapter 3 (see section 3.3.1), where the cell concentration range of interest was concluded to be $20x10^6$ - $100x10^6$ cells/mL. Robust performance at low viabilities was also identified to be a desirable function for a robust primary recovery step. Low viability has been defined in the literature to be <50%, where accurately detecting viabilities below 40% is increasingly difficult (Immarino *et al.,* 2007). Low viability conditions in the previous chapters have been defined as ≤40%. This will therefore continue to be the definition of low viability, and high viability conditions will be defined as ≥90%.

TFF performance in terms of yield has been a concern especially in cases of high concentration of protein, specifically product. The concentration factor which can be achieved by a TFF system as well as the yield are largely limited by the system dead volume. Microfiltration membranes are designed to allow product transmission through the membrane to the permeate, however product loss can still occur by product retention on the membrane, or by non-specific binding to the cell matter in the retentate. Product loss can be decreased by washing the retentate with additional buffer and recovering unbound product remaining in the retentate (Rao *et al.,* 2012). For the purposes of a base level assessment, product yield will be calculated without the implementation of any yield improvement techniques to provide a baseline for each TFF technology performance.

The cell culture variables most likely to impact TFF have been identified to include cell concentration (solids load), and particle size profile (determined by cell viability). As TFF performance can be described by Darcy's Law (see section 1.3.3), fluid viscosity can decrease flux through the membrane, thus negatively impacting on the TFF performance.

Any increase in fluid viscosity can be caused by an increase in protein concentration (including the product) due to increased protein production or a decrease in cell viability. The worst case scenarios discussed in the previous chapters have involved high titre, high impurity cases. Therefore titre and host cell protein concentrations will also be included in the design of experiments as separate factors.

This chapter aims to describe the performance and COG achieved by Bio-Optimal MF-SLTM and QSD™ as a function of key cell culture variables, where the data can be used to identify cell culture conditions which would necessitate a change in technology. Measured responses were selected to capture key metrics indicating TFF performance. These involved throughput as well as product concentration in the permeate and HCP concentration in the permeate. Significant factors were identified and correlations to link these factors to the responses were built using Design Expert 8.0 Software. The correlations for each technology type were incorporated into the economic model described in Chapter 5 (see sections 5.2.4 and 5.3.4). COG values for a range of cell concentration and product titre scenarios were calculated at the 2,000 L scale. The COG values were benchmarked against worst case performance results predicted using experimental data obtained for the centrifugation and depth filtration (30ZA) combination. The most cost effective technology option for each cell concentration and product titre combination was calculated to allow for a formation of a technology strategy for given aims of cell culture performance in terms of cell concentration and titre combinations.

6.2 Materials and Methods

6.2.1 Experimental Overview

Technology options selected based on the screening study and economic analysis described in Chapter 5 were, including QSD™ and Bio-Optimal MF-SL™ carried forward into a centre composite four factorial DoE design in order to obtain correlations relating key cell culture variables to the technology performance outputs (see Figure 6.1). These correlations along with assumptions described in Chapter 5 (see sections 5.2.4) were used to perform a detailed economic assessment and map the COG for the technology options across the selected ranges of cell culture inputs.

Key cell culture variables with the potential impact on tangential flow filtration operation performance were identified to include cell concentration, viability, cell culture titre and HCP concentration. These were selected as the factors for the DoE design further described in section 6.2.2. Cell culture material with the required variable combinations was generated using a 70 L SUB prepared as described in see section 2.2.3. The CCTM material was then processed using QSD™ and Bio-Optimal MF-SL™.

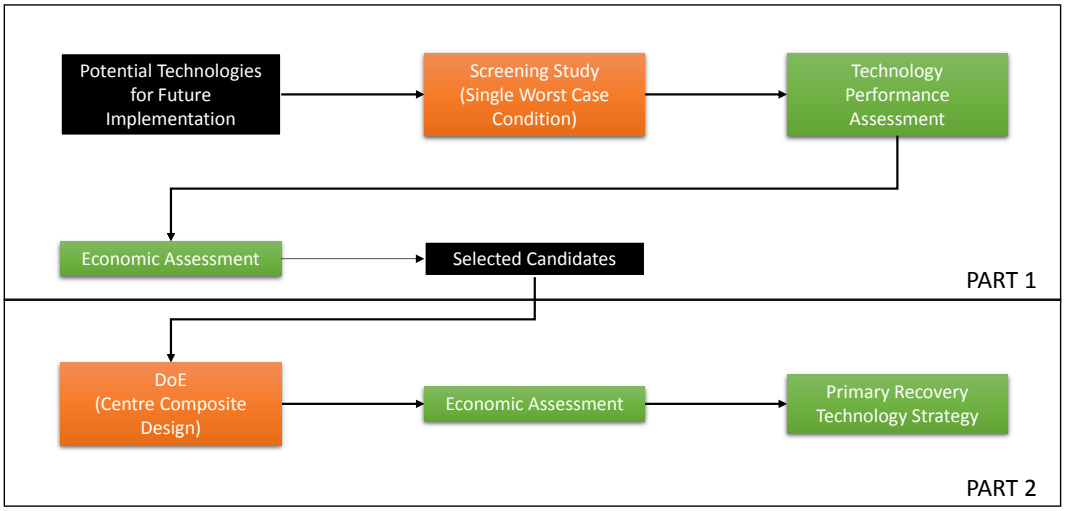

Figure 6.1: *Overview of the integrated experimental and economic framework for primary recovery technology strategy formation. Part 1 of the methodology was described in Chapter 5 providing selected technology candidates including centrifugation and 30ZA, Bio-Optimal MF-SL™ and QSD™ options. This chapter will describe Part 2 of the experimental framework presenting results for the primary recovery technology strategy.*

6.2.2 Design of Experiments Approach

A centre composite approach for a four factorial design, where the factors included cell concentration in the range of 20-100x10^6 cells/m, IgG_1 titre (5-20 g/L), HCP concentration (4,000-7,000 µg/mL) and cell culture viability (90-40%) (see Table 6.1). The experimental runs were ordered in decreasing order of viability, thus allowing the production of material for the entire set of experimental runs using a single 70L SUB cell culture run.

Table 6.1: *DoE design target value inputs used for Bio-Optimal MF-SL™ and QSD™ experiments. These were carried out during the course of one 50 L STR cell culture and therefore were not randomised but were placed in descending order of viability conditions. A model verification point was randomly selected. This data point was not used for model generation, however was obtained in the same cell culture run as the remaining 23 run conditions.*

Run	Cell concentration (x10^6 cells/mL)	Titre (g/L)	HCP Concentration (µg/mL)	Viability (%)
1	100	20	7,000	90
2	20	20	4,000	90
3	100	5	4,000	90
4	20	5	7,000	90
5	60	12.5	5,500	65
6	60	12.5	5,500	65
7	60	12.5	5,500	65
8	60	12.5	5,500	65
9	100	5	7,000	40
10	20	20	7,000	40
11	100	20	4,000	40
12	20	5	4,000	40
Model Verification Point	40	7	5000	50

During the course of the cell culture 2 L of material per day were harvested through the harvest line of the 70 L SUB between day 6 and day 14 of the fed-batch culture. The harvested cell culture broth was then processed to produce a CCTM corresponding to the experimental run conditions described in Table 6.1 using the CCTM methodology described in section 4.2.

Due to the high concentration of the CCTM material produced, large dilution factors were required to obtain cell number and viability readings when using the ViCell™. Thus resulted in a higher level of variability in readings than would be typically expected. Therefore percentage solids readings were also obtained for the starting material and related to the total cell concentration readings. The percentage solids for each experimental run was ultimately used as an input for DoE analysis in order to reduce noise within the analysis. However, throughout this chapter all inputs and correlations obtained through this analysis are related back to total cell concentration (see Figure 6.2), as this is a more relevant metric to describe mammalian cell culture properties.

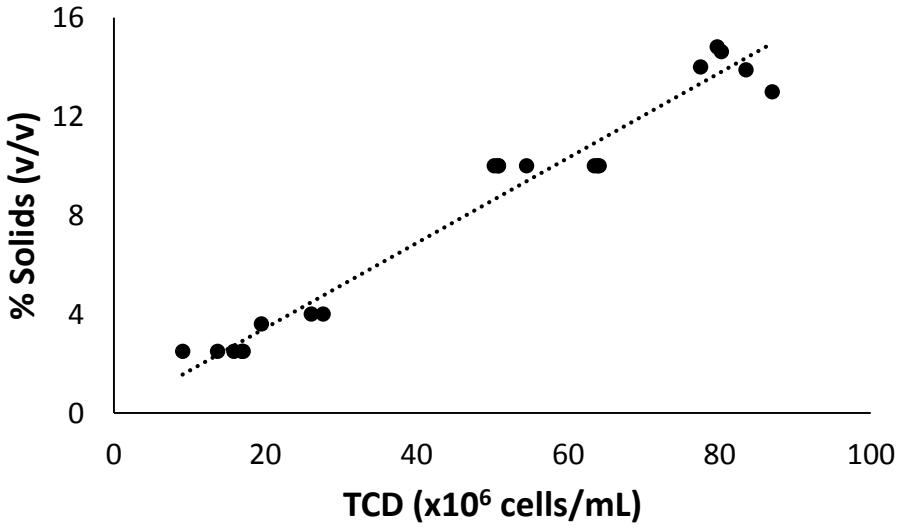

Figure 6.2: *Total cell concentration (TCD) measured using the ViCell™ versus % Solids (v/v) measured using centrifugation of a 5mL cell culture volume. This standard curve was generated and used to convert TCD inputs to Design Expert 7.0 Software to obtain equations for each response. All economic result outputs were converted back to TCD to provide a more comprehensive set of results.*

Twelve small scale modules of Bio-Optimal MF-SL™ and QSD™ were prepared individually by wetting the membrane using purified water for a period of 30 minutes. Water flux of each membrane was then tested at a TMP of 0.1 bar and membrane resistance was calculated for each module and compared to ensure comparability between the modules. Initial cell culture volumes of 43 mL and 65 mL were used for Bio-Optimal MF-SL™ and QSD™ respectively. These were calculated based on a maximum throughput for both modules of 75 L/m^2 and a yield of 70%. The dead volumes for Bio-Optimal MF-SL™ and QSD™ were measured to be 13 mL and 19.5 mL respectively. The modules were run as described in section 2.3.3. Permeate fractions were obtained and OD was monitored to ensure no breakthrough occurred. IgG_1, HCP and DNA concentration was measured as well as the throughput through the membrane. The responses were analysed using the Design Expert 9.0 Software, where the correlations obtained were used to predict the model verification point conditions (see Table 6.1). These were compared to the results obtained in practice.

Post result analysis of the responses, the correlations obtained were used to model IgG_1 and throughput results as a function of the significant factors identified in the analysis. The inputs and the correlations were incorporated into the economic model used in Chapter 5 (see section 5.2.4). COG outputs were calculated for Bio-Optimal MF-SL™ and QSD™ for a single scale scenario of 2,000 L, as this would most appropriately represent a maximum likely scale of operation for high cell concentration, high titre processes. Economic model assumptions are described in section 5.2.5.

6.3 Results and Discussion

6.3.1 50L Cell Culture Material Generation

Cell culture material was produced at pilot scale (70 L) using the previously described in section 2.2.3 . Cell growth, viability profiles were compared to the previously discussed cell culture data obtained at the 5 L and 70 L scales (see Figure 4.2). Cell growth during the second run of the 70 L SUB was kept within 10% of the standard deviation of the 5 L scale and the previous 70 L results (see Figure 6.3). Cell viability was kept within standard deviation of the 5 L and the 70 L scale result obtained previously. A 10% higher maximum cell count was achieved at the same running conditions which may indicate a higher average cell count at the 70 L scale or a greater batch-to-batch variability. In order to determine reasoning for this event a higher number of experimental runs will be required.

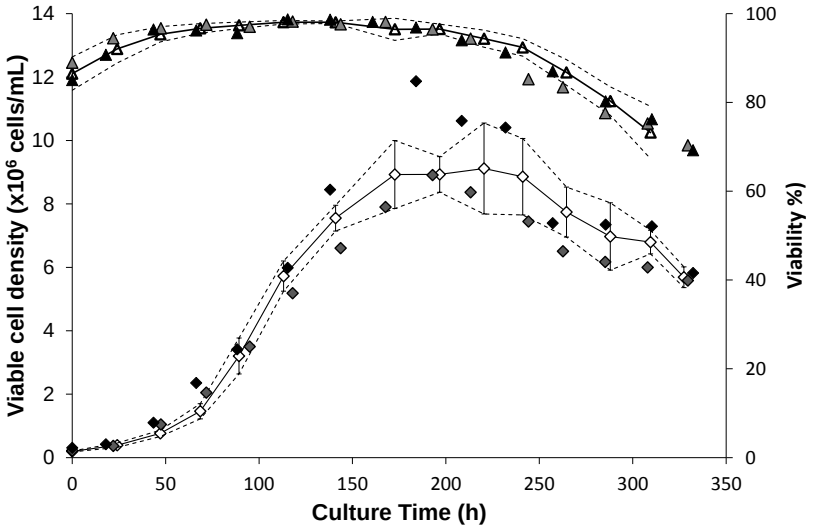

Figure 6.3: *Cell culture growth comparison at 5 L scale and 70 L scales. An average of 5 cell cultures at 5 L scale using BIOSTAT B-DCU glass STRs is shown, where viable cell concentration is shown using ◇ ;viability using △; and the standard deviation is indicated using ----- . Two repeat 70 L cell cultures were carried out using the 70 L CultiBag STR SUB where the viable cell concentration of run 1 is indicated using ◈; and run 2 ◆ ; viability run 1 ▲ and viability run 2 ▲.*

6.3.2 Models for TFF performance based on cell culture conditions using DoE

One TFF module was used per experimental condition. Therefore twelve of each type of TFF modules were compared in terms of water flux prior to use. Membrane resistance between the modules used for the study were found to be within 9% and 25% for the Bio-Optimal MF-SL™ and QSD™ respectively. The two-fold higher variability in the QSD™ modules is most likely due to module number 1 originating from a different lot of membrane

material, therefore the variability includes batch-to-batch variation, whereas the Bio-Optimal MF-SL™ modules originated from the same manufacturing lot.

Bio-Optimal MF-SL™ performance was analysed with respect to cell concentration, viability IgG_1 titre and HCP load (see Table 6.2). Design Expert 8.0 was used to determine the significant factors affecting each measured response, including throughput, IgG_1 concentration in the permeate, HCP concentration in the permeate. Significant factors for throughput through the membrane were found to be total cell concentration (converted to % solids) and cell viability, where throughputs of over 85 L/m^2 were possible at the 2-4% solids (equivalent to 10-20x10^6 cells/mL) range at cell viabilities of 70-90% and 20L/m^2 at the worst case 20% solids (equivalent to 90-100 x10^6 cells/mL) and <40% viability (see Figure 6.4). ANOVA results for the throughput model indicate high significance of both factors as well as the overall model (see Table 6.3). The normal plot versus residuals, as well as predicted versus actual results are shown in Appendix E.

Cell concentration and viability have been previously identified to be significant in affecting membrane throughput in TFF operations (Stressmann and Moresoli, 2008) and in some cases are set as harvest criteria during cell harvest to ensure robust performance of the primary recovery step. Typical filtration sizing techniques include a safety factor which is applied to the required membrane area for a given scale. Cell concentration was found to have a 10 fold effect on the decrease in throughput compared to cell viability (see Table 6.2). Therefore for a specific process and cell line, where a range of cell concentration at harvest is within ±5x10^6 cells/mL from the average typically obtained, cell viability is not predicted to impact throughput significantly, in cases where membrane area has been sized using a safety factor of >40%. For tightly controlled processes where cell viability is not expected to fall below 60% membrane performance is not predicted to be effected when a safety factor of >20% is used.

Initial IgG_1 and HCP concentrations in the CCTM were found to be significant factors in determining their respective concentrations in the permeate (see Table 6.3). The percentage of product and HCP transmitted through the Bio-Optimal MF-SL™ membrane under the explored set of conditions was constant at each of the starting concentrations, independent of cell concentration or the starting cell culture viability. Results were also compared to a control sample, which showed no significant decrease in IgG_1 or HCP concentrations, thus suggesting potential protein adsorption to the membrane, or retentate material. Due to the high concentration and apparent cell damage of the retentate material, methods used for protein and product quantification were deemed insufficient to carry out a mass balance on the protein and product concentrations. In the scope of this study moderate but not optimised process conditions were selected in order to maintain constant flux and shear during processing. In the case that Bio-Optimal MF-SL™ is selected for primary recovery operation, further optimisation work will be required to increase product transmission through

the membrane. Both factors and models generated for these responses were shown to be significant based on ANOVA results given in Table 6.5 (for plots of residuals as well as predicted versus actual results see Appendix E).

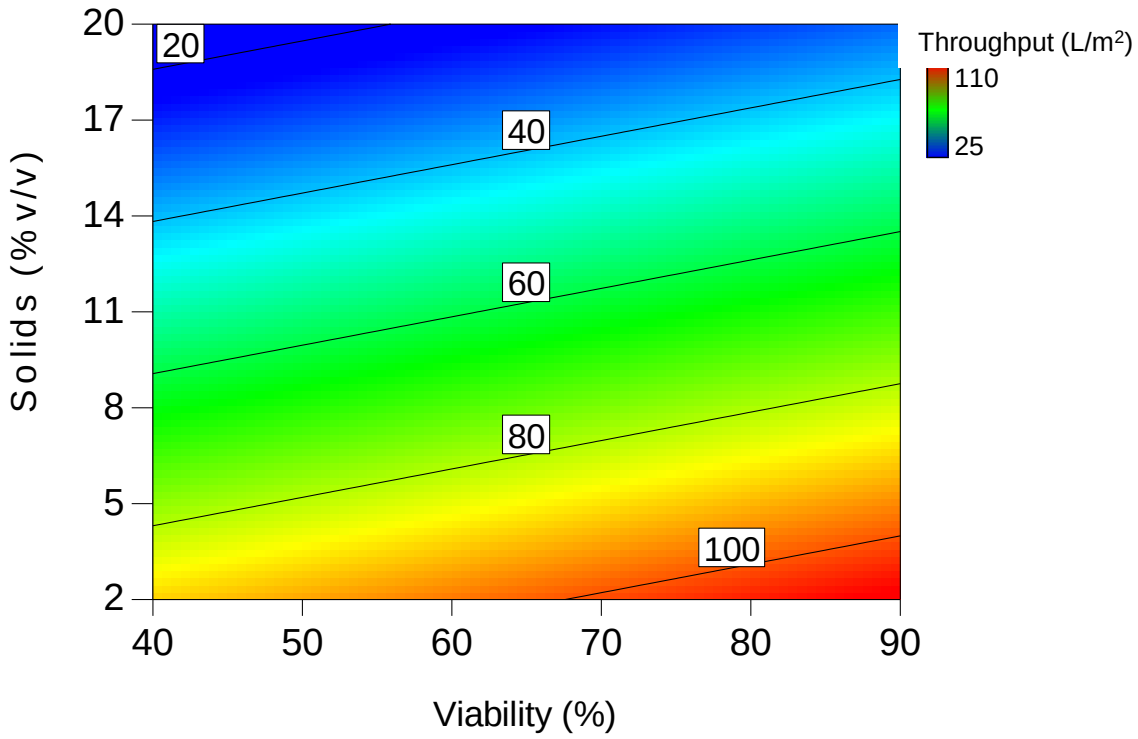

Figure 6.4: *Contour plot of the two factors %solids and cell viability which effect throughput of the Bio-Optimal MF-SL™. Actual % solid and viability values of the feed material to the Bio-Optimal MF-SL were used as opposed to the target conditions. Average values calculated from triplicate measurements using the ViCell™ were used for viability and volume measurement pre and post centrifugation was used to obtain % solids.*

Table 6.2: *Correlations each of the responses calculated using the DoE design applied to the Bio-Optimal MF-SL™. The equations were used to predict Bio-Optimal MF-SL™ performance at given cell culture conditions within the range used to generate the correlations.*

Responses for Bio-Optimal MF-SL™	Equation Used to Predict Performance
Throughput(L/m^2) =f(TCD, Viability)	Throughput = 83-4.2x(%Solids)+0.4x (%Viability) Cell concentration (cells/mL) = %Solids/0.172
$[IgG1]_{Permeate}$ (µg/mL)=f(Titre)	$[IgG]_{Permeate}$=472+0.6x(Titre)
$[HCP]_{Permeate}$ (µg/mL)=f([HCP]	$[HCP]_{Permeate}$=1707+0.6x[HCP]

Analysis of the QSD™ module results in terms of throughput identified cell viability as a single significant factor (see Table 6.4). Maximum throughput achieved using this module at high viability of ≥90% was 90L/m^2, decreasing to a minimum of 4L/m^2. Although filter capacity can depend on solids load, pore blocking mechanisms also vary depending on feed particle size distribution (van Reis and Zydney, 2001). A decrease in cell viability during filtration typically causes a decrease in the average particle size, which may cause increased pore blocking at this membrane size (0.2µm), to a greater degree than in the case of the Bio-Optimal MF-SL™ (0.45µm). However, to explore this further membrane pore size should be included as a factor in the experimental design. The throughput achieved by the QSD is lower than typically advertised by TFF technology suppliers (140-200L/m^2) (Mehta, 2006). However, the reported figures were generated using 10 fold lower cell concentrations, and therefore are not representative in this case.

IgG_1 and HCP concentrations in the permeate were found to be dependent on the starting concentration of the respective components, as in the case of Bio-Optimal MF-SL™. However, transmission of HCPs and the IgG_1 was found to be greater (70% as opposed to 60% for the Bio-Optimal MF-SL™) and also independent of the starting concentration. This may be due to the discussed potential difference in the pore blocking mechanism or the membrane material. Both models were found to be significant with P values of <0.001 (see Table 6.5). See Appendix E shows plots of actual results versus predicted as well as the plots of residuals for each factor.

Table 6.3: *Summary of ANOVA results for the selected model best fitting the results for each of the given responses for both the Bio-Optimal MF-SL™ and QSD™. All selected models as well as the factors within each model are significant where significance was defined by a Prob>F value of <0.05.*

Response	Term	Prob > F	Term	Prob > F
Throughput (L/m2)	Model	<0.0001	Model	0.0131
	Solids Load (%)	<0.0002	Viability (%)	0.0131
	Viability (%)	0.0373	Residual	
	Residual			
[IgG1] in Permeate (µg/mL)	Model	<0.0001	Model	<0.0001
	Titre (µg/mL)	<0.0001	Titre (µg/mL)	<0.0001
	Residual		Residual	
[HCP] in Permeate (µg/mL)	Model	<0.0001	Model	<0.0001
	[HCP] in Feed (µg/mL)	<0.0001	[HCP] in Feed (µg/mL)	<0.0001
	Residual		Residual	

Table 6.4: *Correlations each of the responses calculated using the DoE design applied to the QSD™. The equations were used to predict QSD™ performance at given cell culture conditions within the range used to generate the correlations.*

Responses for QSD™	Equation Used to Predict Performance
Throughput(L/m^2) =f(Viability)	Throughput = 9.3+0.98x(%Viability)
$[IgG_1]_{Permeate}$ (µg/mL)=f(Titre)	$[IgG_1]_{Permeate}$=-102+0.7x(Titre)
$[HCP]_{Permeate}$ (µg/mL)=f([HCP]	$[HCP]_{Permeate}$=740+0.7x[HCP]

The correlations derived using experimental data for each factor were tested using the predictive function of the Design Expert 8.0 software and the data obtained for the model verification point during the course of the experimental run. Models used to describe the Bio-Optimal MF-SL™ were found to be within 21% of the experimental data within the range of the model (see Table 6.5). The QSD™ model was found to be within 15% of the experimental data, where the highest variability was seen in the HCP transmission data. One of the limitations of the implemented design is its inability to check for curvature in the area outside the tested points. Although centre points are present in both designs, some unknown curvature may still be present within the described area and could be a potential cause for the higher percentage variation values e.g. throughput and $[HCP]_{Permeate}$ in the case of Bio-Optimal MF-SL™ or $[HCP]_{Permeate}$ in the case of the QSD™.

Table 6.5: *Summary of the experimental Vs model predicted values for the Bio-Optimal MF-SL™ and QSD™ models calculated for each of the selected responses.*

Bio-Optimal MF SL™ Responses	**Experimental versus Predicted (%)**
Throughput (L/m^2)	21
[IgG] Permeate (µg/mL)	0.5
[HCP] Permeate (µg/mL)	25
QSD™ Responses	**Experimental versus Predicted (%)**
Throughput (L/m2)	1
[IgG] Permeate (µg/mL)	1
[HCP] Permeate (µg/mL)	15

6.3.3 Economic Assessment

In order to analyse the potential of the selected technologies for future primary recovery operation the two identified TFF technologies were assessed on their economic viability by modelling their performance at the 2,000L scale of operation, at the cell concentration, product titre, viability and impurity concentration ranges expected to be reached in the future. Correlations derived from the experimental data, described in section 6.3.2 were used to calculate membrane sizing requirements in each cell culture scenario in conjunction with assumptions described in sections 5.2.4 and 5.2.5 to complete the economic assessment.

COG generated for the Bio-Optimal MF-SL™ and QSD™ were compared to the COG generated by the centrifugation and 30ZA depth filtration model, where inputs included the 20-100x10^6 cells/mL cell concentration range and low cell viability scenarios. COG ratios of the TFF option to centrifugation and depth filtration option were calculated for each of the alternative technologies at 40% cell viability centrifugation and 30ZA option (see Figure 6.5).When comparing the COG values and throughput for centrifugation & 30ZA to Bio-Optimal MF-SL™, the Bio-Optimal MF-SL™ showed a higher COG than centrifugation &30ZA option for cell concentrations below 60x106 cells/mL, however, for cell concentrations >60x10^6 cells/mL Bio-Optimal MF-SL™ was the more economically viable option. The QSD™ COG was 3-4 fold higher than that predicted for the centrifugation & 30ZA option at cell concentrations below 50x10^6 cells/mL and 2-fold higher at 50-80x10^6cells/mL. However, the option becomes economically viable once cell concentrations exceed 80x10^6cells/mL. In both cases centrifugation and 30ZA COG becomes increasingly less efficient with increasing cell concentration, due to the yield losses encountered as a result of solids discharge during centrifugation. The centrifuge used to model yield losses for the 2,000L scale of operation was selected to represent an average current design (Alfa Laval BTPX-205). However, alternative centrifuge designs which may be implemented in the future could significantly reduce the yield loss at this stage e.g., KSep technologies may provide an alternative to the classic solids discharge mechanisms potentially allowing for a reduction in yield loss (Ko and Bhatia, 2012).

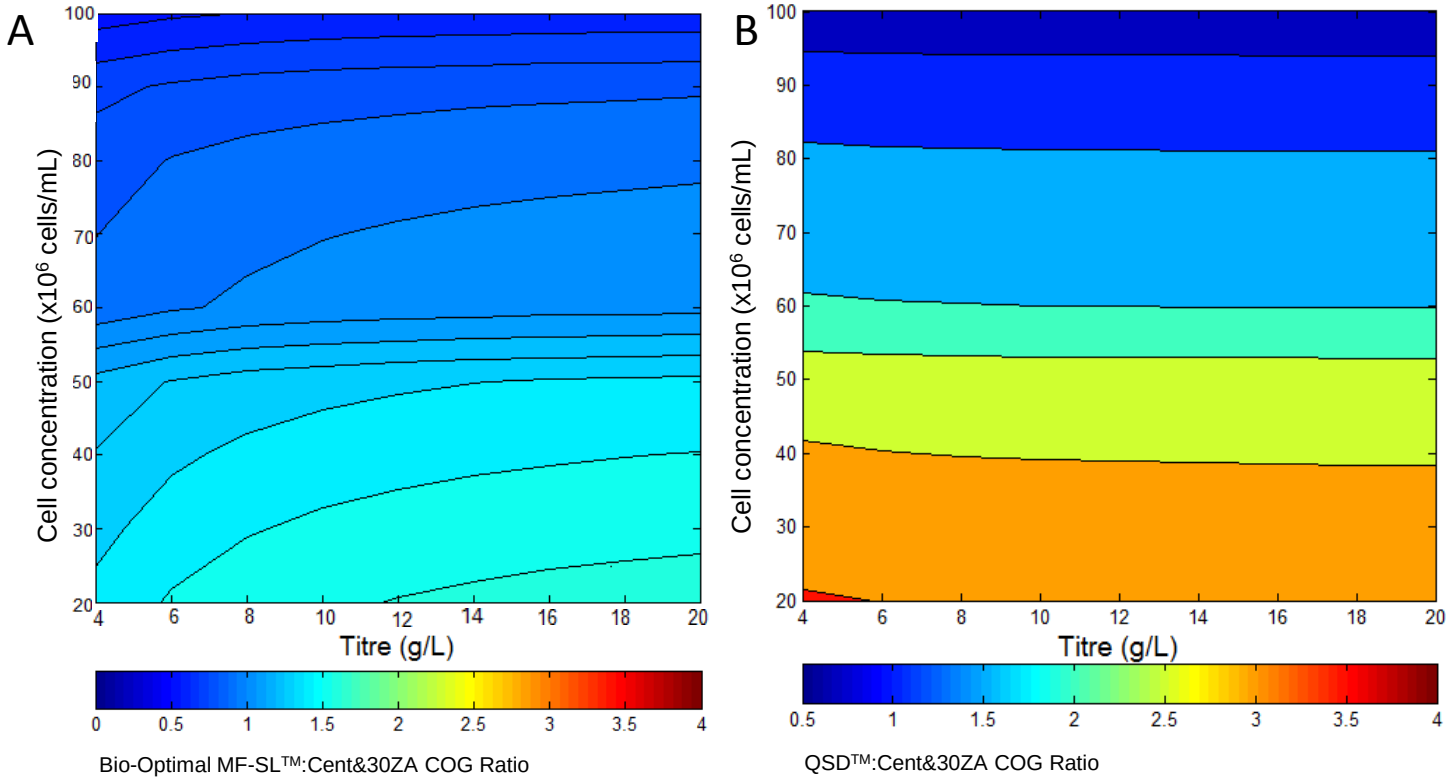

Figure 6.5: *Calculated COG ratios for 40% cell viability, the explored range of cell concentrations and titre at the 2,000L scale comparing A: Bio-Optimal MF-SL™ : Centrifugation & 30ZA option and B: QSD™ : Centrifugation & 30ZA option.*

Bio-Optimal MF-SL™ and the QSD™ COG was compared for a range of cell viabilities at a fixed cell concentration of $60x10^6$ cells/mL (see Figure 6.6). The impact of viability on the Bio-optimal MF-SL™ COG is lower than that on QSD™ COG. The material costs of the QSD™ modules was 2-fold higher (see section 5.2.5). Hence, any impact on performance arising as a consequence of an increase of the surface area results in a significant increase in COG compared to the Bio-Optimal MF-SL™ option. In order to achieve equivalent COG for the Bio-Optimal MF-SL and QSD modules a cell culture process may be operated at a product titre of >14g/L for the Bio-Optimal MF-SL and a titre of 10-14g/L and a viability of >60% or a titre of 18-20g/L at the low viability range of 30-40%.

Titre increases are typically achieved via a combination of cell concentration and cell specific productivity increases (see section 3.3.1). The lowest cost primary recovery options for the combinations of cell concentrations and titres possible to achieve in the future were calculated for the worst case viability of 40% (see Figure 6.7). All options with COG values within 0.05 RMU/g were stated as viable for a given specific cell concentration and viability combination. Centrifugation and 30ZA option has the lowest COG for harvests of cell concentrations below $60x10^6$ cells/mL. The Bio-Optimal MF-SL™ data matches the COG of the centrifugation and 30ZA option between $60x10^6$ and $80x10^6$ cells/mL, however for titres of <8g/L Bio-Optimal MF-SL™ is the lowest cost option. At cell concentrations of $>80x10^6$ cells/mL and titres of >12g/L the QSD™ option matched the Bio-Optimal MF-SL™ COG, making both options equally viable.

Current cell culture harvest strategies tend to specify harvest criteria in terms of cell viability to ensure the success of the primary recovery step to follow as well as other reasons which may be product or cell line specific (e.g. product stability). It may therefore be argued that the QSD™, although sensitive to changes in cell viability could act as a future proof robust option, as its performance was least sensitive to changes in cell concentration. For cases where a change in technology is significantly less desirable than the cost of the primary recovery step, QSD™ may provide a single technology option which will be viable across the cell concentration and titre ranges explored in this study.

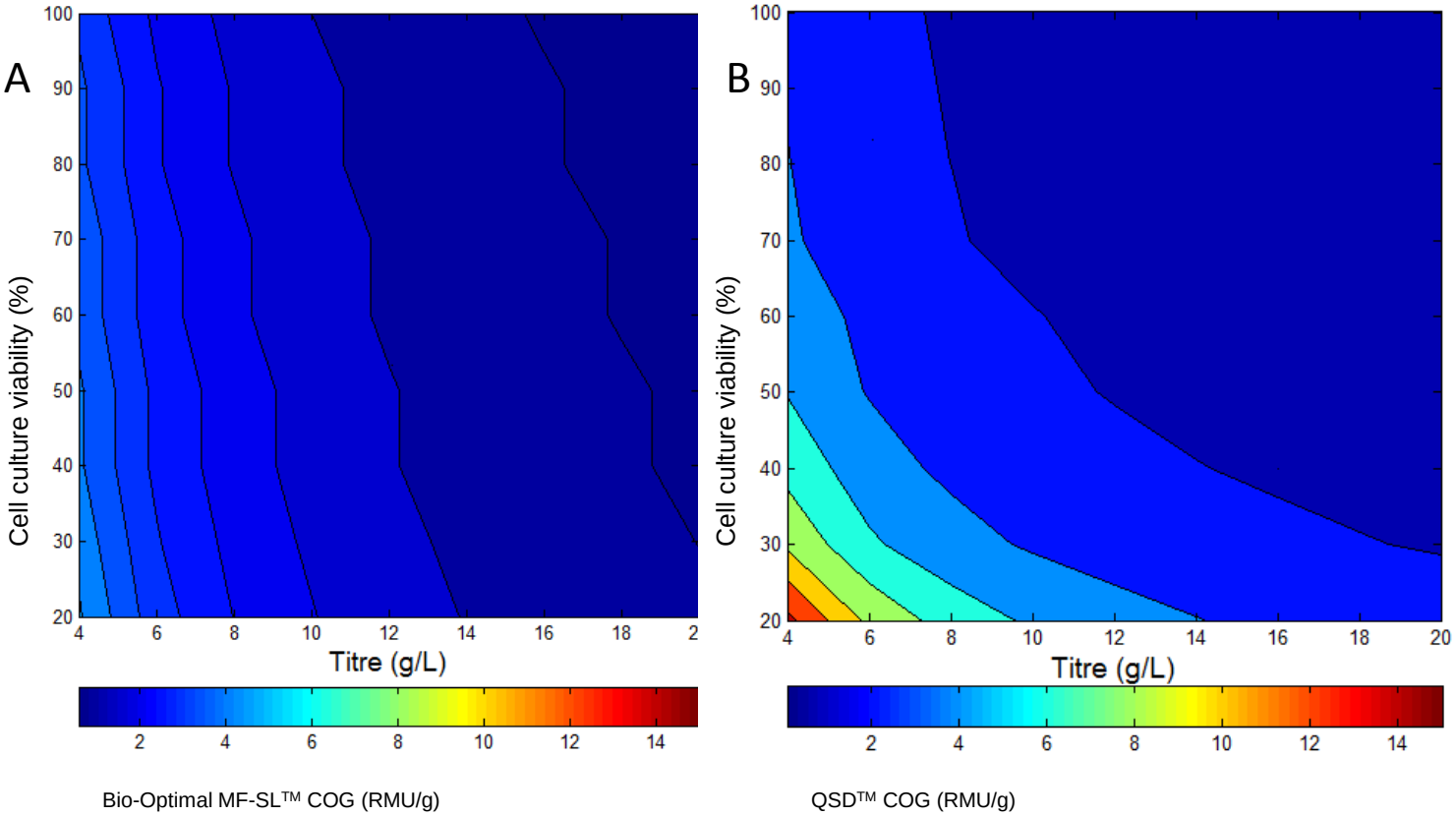

Figure 6.6: *Cost of goods (RMU/g) modelled for a range of cell viability and titre scenarios at the 2,000L scale for a 60x10^6 cells/mL cell concentration scenario. A: Bio-Optimal MF-SL™; B: QSD™.*

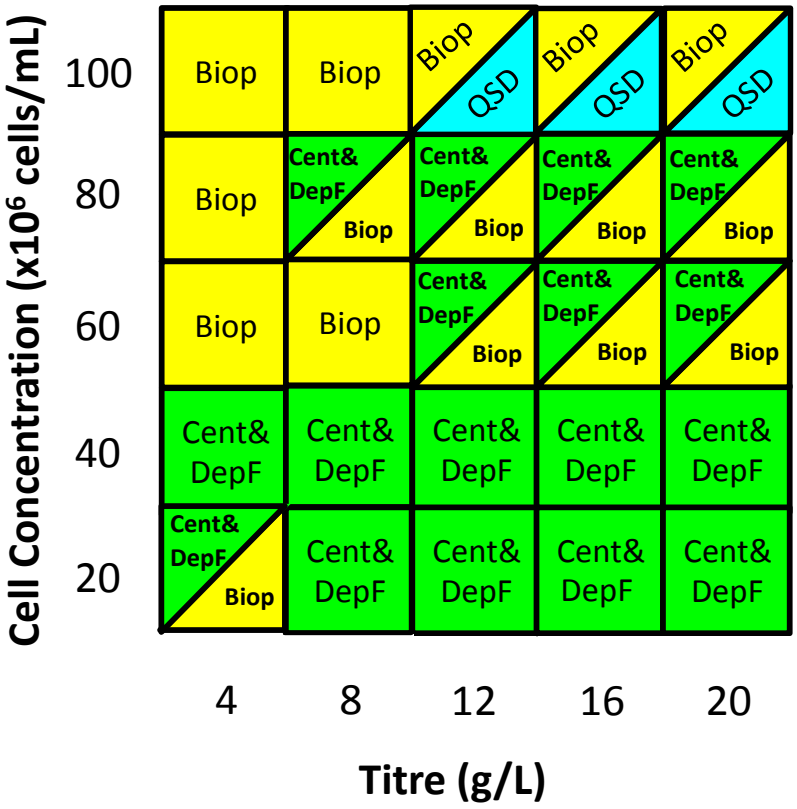

Figure 6.7: *Summary of economically viable technologies at the given cell concentration and titre ranges. Economically viable technology is defined as the lowest COG at the given scenario, all viable options within a 0.05 RMU/g range are shown for each scenario.*

6.4 Conclusions

This final chapter has presented a second successful scale up run of the 70 L Sartorius Cultibag™ single use bioreactor. Peak viable cell count during the course of the batch exceeded the expected cell count obtained at the 5 L scale, however more runs would be required to determine whether a higher average cell concentration is achieved consistently at the 70 L scale.

The material generated at the 70L scale produced cell culture test material for a DoE study used to identify correlations for the significant key cell culture variables which affect Bio-Optimal MF-SL™ and QSD™ performance. The correlations were than used to calculate COGs for combinations of cell concentration, titre and impurity concentrations for conditions currently seen in mammalian cell culture and those expected over the next decade (2,000 L scale scenario).

Bio-Optimal MF-SL™ throughput performance was found to be affected by a combination of total cell concentration and viability, where cell concentration had a fourfold higher impact on throughput and impacted directly the membrane area required. IgG_1 and HCP concentrations in the permeate were independent of cell concentration or viability and depended on starting protein concentration in both cases of the product and impurity, where 60 % of the starting HCP and IgG_1 concentrations were found in the permeate. QSD™ throughput performance was directly dependent on cell viability and did not correlate significantly with cell concentration. However further experiments would be recommended to confirm the absence of curvature in the explored design space. 70% of HCP and IgG_1 was found to be present in the permeate, suggesting lower potential product loss in the case of QSD™ when compared to the Bio-Optimal MF-SL™. All found correlations were tested using randomly selected experimental conditions within the tested range. All of the QSD™ and Bio-Optimal MF-SL™ correlations were found to be within 25% of the predicted range.

COG was calculated for the two alternative TFF options at the full range of cell concentration, viability and titre combinations tested and benchmarked against the COG calculated for the centrifugation and 30ZA option. The comparison showed the centrifugation and 30ZA option to be the most cost effective across the titre range at cell concentrations $<50x10^6$ cells/mL For the Bio-Optimal MF-SL™ option it was equally cost effective at cell concentrations of $60\text{-}90x10^6$ -$80x10^6$ cells/mL and more cost effective than the centrifugation and depth filtration option at titres less than 8g/L. Once cell concentrations exceed $80x10^6$ cells/mL the Bio-Optimal MF-SL™ was most cost effective at the lower titre ranges and QSD™ becomes equally as cost effective once titres exceed 12 g/L. Finally, it may also be argued that the single future proof technology, independent of cost would be the QSD™ as its performance is not dependent on cell concentration, and therefore would provide a robust option for processes where the harvest viability is controlled.

Implementing the use of a new technology not previously employed in the process is classed as a major process change. Obtaining approval for such a change will typically require a substantial

validation and supporting data package. The next chapter will discuss the validation considerations involved when implementing these sets of changes to existing processes.

Chapter 7: Validation Considerations in Implementation of Major Process Changes to Exiting Processes

Process validation strategies as well as validation of process changes have been driven towards the implementation of continuous improvement through risk management and quality by design approaches since the publication of the “Guidance for Industry- Process Validation: General Principles and Practices” by the FDA (Scott, 2011). In addition, the new guidance documents produced by the International Conference on Harmonisation Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) address process validation issues in ICH Q7 (Good Manufacturing Practice), ICH Q8 (Pharmaceutical development) and ICH Q11 (Development and Manufacturing of Drug Substances). These guidelines provide a basis of drug registrations in the EU, US and Japan, and highlight that qualification efforts alone are no longer sufficient without established process control over the course of the process lifetime.

Changes to existing manufacturing processes can include minor, moderate and major changes recording categories. A major change carries the potential to adversely affect the identity, strength, quality, purity or potency of a drug product. Making such a change requires approval from the FDA, prior to the distribution of the resulting product material. Thus a “Prior Approval Supplement Nº 314.70(b)” must be submitted. In order to identify the potential impact of the process change, an assessment of the effects of the change must first be carried out. This includes the assessment of the change on the product conformance to specification in terms of the listed acceptance criteria. Once the material is deemed to continue to meet specification, additional testing is recommended to assess the change effect on product identity, quality, strength or effectiveness. During the course of performing the additional testing product equivalence of the product before and after the change must be demonstrated.

Currently, the manufacturing costs of biopharmaceutical products are far outweighed by the R&D costs involved in the search for a successful drug candidate and the manufacturing process continues largely to define the drug product, despite drive towards the QbD approach. This means that risk can be carried through to GMP drug manufacture stages in cases where high uncertainty within the processing strategy exists.

As primary recovery and purification operation performance can be highly dependent on cell culture, it becomes very important to ensure the robustness of the downstream strategies. One of the ways in which this could be achieved is by developing a highly robust primary recovery stream which can cope with the likely variance produced by the variability in cell culture operations, and consequently keep the purification of the resultant product stream functioning consistently and reliably.

This thesis has discussed primary recovery strategies in the context of implementation in a contract manufacturing organisation (CMO), producing a range of biological products for a wide range of companies from small biotech to big pharma. Typically, production contract lengths and production scale may vary; some customers may only need production for one Phase of a clinical trial, and may want to transfer the process to their own facility at a later stage, whereas other projects may require large scale manufacture.

Chapter 6 identified two tangential flow filtration options for primary recovery with the potential to provide cost effective technology options for the future. The validation package required for their implementation in place of a current processing options would depend on whether they are applied as a single use or reusable technology. Single-use equipment is increasingly recognised as an advantageous option for CMO use (Fuller and Pora, 2008), however, re-using the TFF technologies up to 5-10 times significantly reduces the COG (see section 6.3.3).

Assuming that the selected TFF technology will be re-used up to 10 times, cleaning validation studies will be required. In addition, reusability studies of the TFF option at small scale would be required as well as a validation at large scale. In addition, the TFF modules used for the purpose of the studies described in this thesis, have been designed to be sterilised using a 70% ethanol rinse cycle and hold. Steam sterilisation cannot be used due to the adhesives in the construction of the modules not being able to withstand the sterilisation temperature. This may be considered less reliable to autoclaving, and require a more extensive validation data package.

Re-using the TFF filters would require leak testing and filter integrity testing to be verified at end of each batch run which would increase the post processing activity times. Having to carry out a leak or integrity testing at the end of each run introduces an additional contamination risk by introducing additional solution and material handling stages. Although leachables and extractables testing is associated with single-use equipment, the validation effort associated with this may still apply to this unit operation as safety will still have to be demonstrated.

Three to four pressure sensors would be required for a reliable module operation during processing, which are not currently a part of the system. These would also have to be installed and verified prior to use. The key pressure sensors required for operation include the feed and the permeate pressure sensors, without these being fully functional the unit operation cannot proceed reliably. A plan to mitigate the risk associated with their failure should be included in the control strategy (e.g. a procedure to change pressure sensors during processing, include spare sensors prior to start of a batch etc). Single use pressure sensors could potentially serve as a viable option as they can be purchased as part of the single-use flow path, pre-validated and thus changed after each run.

A separate validation issue which applies to primary recovery operations in general includes defining control point criteria for material release for further processing. As cell culture material can be highly variable, it is important to ensure a consistent output by the primary recovery unit operations to reduce potential batch failure. For example, it may be easier to ensure consistent solids removal specification during primary recovery by introducing appropriate safety factors during filter or membrane sizing, however if impurity removal specification is introduced, it may be more difficult to meet if changes in impurity profile or load are detected. However, if specific changes to the impurity profile or load have been found to influence the Quality Target Product Profile (QTPP) they must be part of the control strategy and included in the control point criteria.

The following section will provide an overview of the conclusions drawn throughout the chapters of this thesis. It will also propose additional future work which would strengthen the arguments presented to date.

Chapter 8: Conclusions & Future Work

The continuing trend of rising mammalian cell culture densities and titres in the biotechnology industry has been increasing the strain on primary recovery operations bringing into question the limit to the current technologies and the search for suitable alternatives for implementation. Choosing a suitable technology for an existing platform process is difficult, particularly with long term robust performance in mind. The overall methodology and results discussed in this thesis have created a structured approach to the testing and selection of primary recovery technologies based on providing robust processing of cell culture material expected over the next decade.

8.1 Overall Conclusions

The first aim of this thesis, described in **Chapter 3**, was to identify current and future demands on primary recovery operations by surveying expert opinion. This survey was carried out successfully in order to quantify the current and expected trends in cell culture, primary recovery and purification operations.

The results of the survey agreed with the literature in confirming that primary recovery and purification constraints have been experienced. Based on this the survey also highlighted the need for impurity removal to be a capability of the desired primary recovery technology as well as solids removal. In addition, a general increase in demand for new technologies across the biotechnology sector was also identified.

Expected titre and cell concentration ranges were also identified for the future cell culture feed to primary recovery based on expert opinion, which was confidence waited and averaged across the surveyed population. Expected titre in 2020 was estimated to be in the range of 10-20 g/L with an expected cell concentration projected to be in the range of 50-100x10^6 cells/mL. The upper limits of these ranges were then used throughout the following chapters as a worst case for the cell culture material that the technologies would face in the future.

Priority criteria for impurity removal targets for the primary recovery technologies were identified. HCP removal was identified as the primary concern, followed by DNA and product aggregate removal.

Chapter 4 aimed to develop and demonstrate an experimental methodology for the creation of a mammalian cell culture test material (CCTM), consistent with the anticipated profile in the next decade. Cell concentration, cell viability, product and impurity concentrations were each manipulated to achieve a range of cell culture test conditions to provide a controlled set of key variables in the cell culture feed to primary recovery. Key cell culture parameters including cell concentration, cell viability, product and HCP concentrations were successfully

decoupled. The CCTM method was found to decouple the selected variables in the cell culture feed to downstream operations, while providing consistent material which is representative of typical cell culture material in terms of key features such as particle size distribution and HCP population. The accurate replication of these features made this methodology suitable for application in primary recovery studies to test the potential for the technologies to cope with the high cell concentration, high titre conditions expected in the future.

Chapter 5 aimed to use a combination of cell culture test materials (CCTM), ultra scale-down technologies, multi-attribute decision-making methods, process economics and facility fit considerations, to demonstrate a methodology and results for achieving a screening of current and alternative primary recovery technologies. The example technologies tested using this method included three centrifugation and depth filtration options (using 05SP, 10SP and 30ZA media), and two alternative tangential flow filtration options (using Bio-Optimal MF-SL™ and QSD™ hollowfibre modules).

MADM analysis as well as selection based on current technology performance criteria showed that only two options met the yield and purity criteria: Bio-Optimal MF-SL™ and the QSD™. The centrifugation and the 30ZA option met the purity criteria but not the yield criteria. The options were further evaluated based on their economic performance. This showed the centrifugation and 30ZA option to be the least cost-effective across the 2,000 L, 10,000 L and 20,000 L scale scenarios and not fit the facility constraints set based on a typical existing large-scale facility. The Bio-Optimal MF-SL™ option was the most cost-effective option across the 2,000-20,000 L scales of operation.

Chapter 6 presented a DoE study which was used to identify correlations for the significant key cell culture variables which affect Bio-Optimal MF-SL™ and QSD™ performance. The correlations were than used to calculate COGs for combinations of cell concentration, titre and impurity concentrations for conditions currently seen in mammalian cell culture and those expected over the next decade (2,000 L scale scenario).

Bio-Optimal MF-SL™ throughput performance was found to be affected by a combination of total cell concentration and viability, where cell concentration had a fourfold higher impact on throughput and impacted directly the membrane area required. QSD™ throughput performance was directly dependent on cell viability and did not correlate significantly with cell concentration. However further experiments were recommended to confirm the absence of curvature in the explored design space. All of the QSD™ and Bio-Optimal MF-SL™ correlations were found to be within 25% of the predicted range.

The COG comparison showed the centrifugation and 30ZA option to be the most cost effective across the titre range at cell concentrations <50x10^6 cells/mL. For the Bio-Optimal MF-SL™ option it was equally cost effective at cell concentrations of 60-80x10^6 cells/mL and more cost effective than the centrifugation and depth filtration options at titres less than 8 g/L. Once cell concentrations exceed 80x10^6 cells/mL the Bio-Optimal MF-SL™ was most cost effective at the lower titre ranges and QSD™ became equally as cost effective once titres exceed 12 g/L. Overall, it was argued that the single future proof technology, independent of cost would be the QSD™ as its performance is not dependent on cell concentration, and therefore would provide a robust option for processes where the harvest viability is controlled.

8.2 Future Work

Proposal 1

CCTM material was shown to be representative of some key features of mammalian cell culture including particle size distribution and the range of host cell proteins it provides. However, the material is expected to be limited in its comparability to true high cell concentration cell culture material, each variable of interest has been individually altered, as opposed to being caused by a realistic scenario. There is scope for a comparative run testing the primary recovery of CCTM material versus high cell concentration material. It is proposed that the high cell concentration material is generated using the same cell line as is used for the CCTM material generation. The CCTM material would be made up to mimic the high cell concentration culture as closely as possible, in terms of cell concentration, titre and HCP concentration. Particle size distributions, HCP populations and titre are recommended to be compared between the two material sets. In addition to this, shear sensitivity of the cells is also recommended to be compared. This is proposed to be carried out using a shear device, which can provide consistent shear for the testing both materials. Particle size distribution prior and post shear exposure should be compared. This would expose any effects of the culture method on cell robustness and subsequently question whether the CCTM should be produced using the same or similar cell culture techniques in order to result in material adequately representative for primary recovery operations.

Proposal 2

Method validation studies are also recommended in order to explore the robustness of the DoE design used to derive correlations for the performance of the Bio-Optimal MF-SL™ and the QSD™ in Chapter 6. Although reliability data for the CCTM material described above would strengthen the case for the use of the correlations, it would not provide sufficient data to show that the use of high cell concentration material from a different origin would not

result in alternative performance profile in either technology. This may be an issue for two factors:

- The use of CCTM versus true high cell concentration material
- The use of an alternative cell line for the generation of the high cell concentration material.

It is therefore recommended to explore both options by using material produced in proposal 1 for the comparison of the Bio-Optimal™ and QSD™ performance at a single cell culture condition. A separate experimental would be required to test the two options using an alternative cell line.

Proposal 3

The identified primary recovery technologies have been assessed based on direct application for cell removal, as well as impurity removal. Achieving impurity removal earlier in the process was aimed at reducing the burden on downstream operations, however this theoretical benefit has not been fully explored. A further study is recommended, aimed at quantifying the impact of the change in the primary recovery step on protein A resin lifetime, as well as impurity removal in the bulk drug product. This would help to create an even more complete analysis of the process consequences of adopting technology advances.

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Appendices

10.1 Appendix A

Mammalian cell processing industry trends and their impact on primary recovery

Daria Popova, EngD Researcher with Lonza Biologics and UCL

Academic supervisors: Nigel Titchener-Hooker & Suzanne Farid

Industrial supervisor: Ashley Westlake

Significant increases in cell culture titres have resulted in higher cell concentrations, product masses and impurity loads. This has increased strain on both primary recovery and purification operations. This is especially a challenge in facilities which have been designed to cope with lower titres and cell concentrations. This survey is part of the UCL–Lonza EngD collaboration on assessing and improving the current performance of primary recovery operations when used in processing of high cell concentration feed streams. The research will greatly benefit from interviews with experts in different processing areas so as to gain a complete and accurate overview of the current and potential future pressures on primary recovery. We are seeking your input to gain insight into the following trends:

- Increase in CMO popularity and its effect on the current capacity at Lonza, highlighting the operating area which experiences most capacity constraints.
- Effect of increases in titre, cell concentration and impurity levels over the recent years
- Consequences of decrease in clarification efficiency
- Future expectations in terms of customer demand and its effect on future processing
- The impact of increasing titres and cell concentrations on the future requirements of each processing area
- Changing future regulatory demands

The survey will be carried out in an interview form and should take no longer than 20-30 minutes. The questionnaire consists of 12 questions, most of which are multiple choice, however discussion and additional comments are most welcome. Any stated figures or values disclosed during the process will remain confidential. During analysis any exact values will be desensitised. If you are interested, the results of the survey can be emailed to you when the study is complete.

FOR EACH QUESTION PLEASE ALSO INDICATE YOUR CONFIDENCE RATING as H/M/L where

H = high confidence based on data/experience,

M = medium confidence based on gut feeling,

L = low confidence based on a guess

1. In production using mammalian cell culture, which processing areas have you experienced operational constraints over the last 2 years?

(You are welcome to tick more than one box)

i. Cell culture ☐

ii. Primary recovery ☐

iii. Purification ☐

iv. Other (Please specify) _____ ☐

v. None have been experienced ☐

Confidence (H,M,L) ☐

2.

a. In your experience have you observed an increasing customer interest in alternative unit operations to those you currently offer?

i. Yes ☐

ii. No ☐

Confidence (H,M,L) ☐

b. If yes, which are most commonly requested?

i. Alternative cell culture options ☐

Please tick if you are referring to perfusion culture ☐

☐

ii. Flocculation options ☐

iii. Precipitation

iv. Cell settling/ sedimentation ☐

v. Expanded Bed Adsorption ☐

vi. Protein A alternatives (Please specify) ☐

_____

vii. Other primary recovery options (Please specify) ☐

_____

viii. Other purification options (Please specify) ☐

_____

Confidence (H,M,L) ☐

3. Do you feel there is pressure to limit cell concentrations due to operational constraints experienced in downstream operations?

i. Yes ☐

ii. No ☐

Confidence (H,M,L) ☐

4. What maximum cell concentrations do you expect to achieve over the next decade?

i. Please Specify _____

Confidence (H,M,L) ☐

5. What maximum mAb titres do you expect to achieve over the next decade?

ii. Please Specify _____

Confidence (H,M,L) ☐

6. What cell culture strategies do you expect will allow you to achieve the higher cell concentrations and titres?

(You are welcome to tick more than one box)

i. Fed- batch stirred tank reactor ☐

ii. Perfusion stirred tank reactor ☐

iii. Alternative media ☐

iv. Alternative feeding strategy ☐

v. Alternative cell line ☐

vi. Other

(Please specify)

_____

Confidence (H,M,L) ☐

7. **Titre increases are usually due to increases in cell concentrations or specific productivities or a combination of both. Please rank the following routes to achieving a *2-fold increase in titre from 4g/L to 8g/L* for a particular cell line in order of likelihood, where 1 is the most likely and 5 the least likely?**

   a. 2-fold increase in c*ell density* ☐

   b. 2-fold increase in *specific productivity* ☐

   c. Increases in both *cell density* and *specific productivity* ☐

Confidence (H,M,L) ☐

8. **What cell density levels could cause operational constraints in these recovery options?**

   (You are welcome to tick more than one box)

		Cell density (cells/mL)						Confidence (H,M,L)
		$3x10^6$	$5x10^6$	$1x10^7$	$2x10^7$	$5x10^7$	N/A	
i.	**Recovery by depth filtration alone**							
ii.	**Recovery by centrifugation & depth filtration**							

9. Assuming the current purification capacity, what % removal of each impurity would be desirable from a primary recovery stage so as to avoid constraints in purification?

(You are welcome to tick more than one box)

		% Removal		
		1-10%	**10-20%**	**20-40%**
i	**HCP**			
ii	**Aggregates**			
iii	**DNA**			
iv	**Other- Please state:**			
v.	**Other- Please state:**			

Confidence (H,M,L) ☐

10. Please rank the following cell culture induced impurities in order of difficulty of removal, where 1 is the simplest to remove and 5 is the most difficult.

		Rank
i	**HCP**	
ii	**Aggregates**	
iii	**DNA**	
iv	**Other- Please state:**	
v	**Other- Please state:**	

Confidence (H,M,L) ☐

11. If no changes are made to purification for the next 5 years, what HCP levels would cause operational constraints in chromatography operations?

(You are welcome to tick more than one box)

	HCP load	**1,000,000 (ng/mg)**					**Confidence (H,M,L)**
	Titre (g/L)	**1**	**2**	**5**	**10**		
	Absolute HCP load (ng/mL)	≤1,000,000	≤2,000,000	≤5,000,000	≤10,000,000	**N/A**	
i.	**Protein A**						
ii.	**AEX**						
iii.	**CEX**						

12. What factors do you believe will lead to increases in HCP levels?

(You are welcome to tick more than one box)

i. Low viability cell culture ☐

ii. High shear during centrifugation ☐

iii. High cell density of the culture ☐

iv. High titre ☐

Confidence (H,M,L)	☐

13.a. With improvements in PAT technologies and assays, do you envisage the regulatory authorities imposing changes to lot release specifications?

i. Yes ☐

ii. No ☐

iii. Don't know ☐

Confidence (H,M,L) ☐

b. If Yes, please indicate what changes may be expected

_____

_____

14.a. With new assays for HCP detection giving higher readings, is it likely to lead to higher demands on recovery and purification?

i. Yes ☐

ii. No ☐

iii. Don't know ☐

Confidence (H,M,L) ☐

b. If yes, please state the key problem you think this would cause for your current processing.

_____

_____

10.2 Appendix B

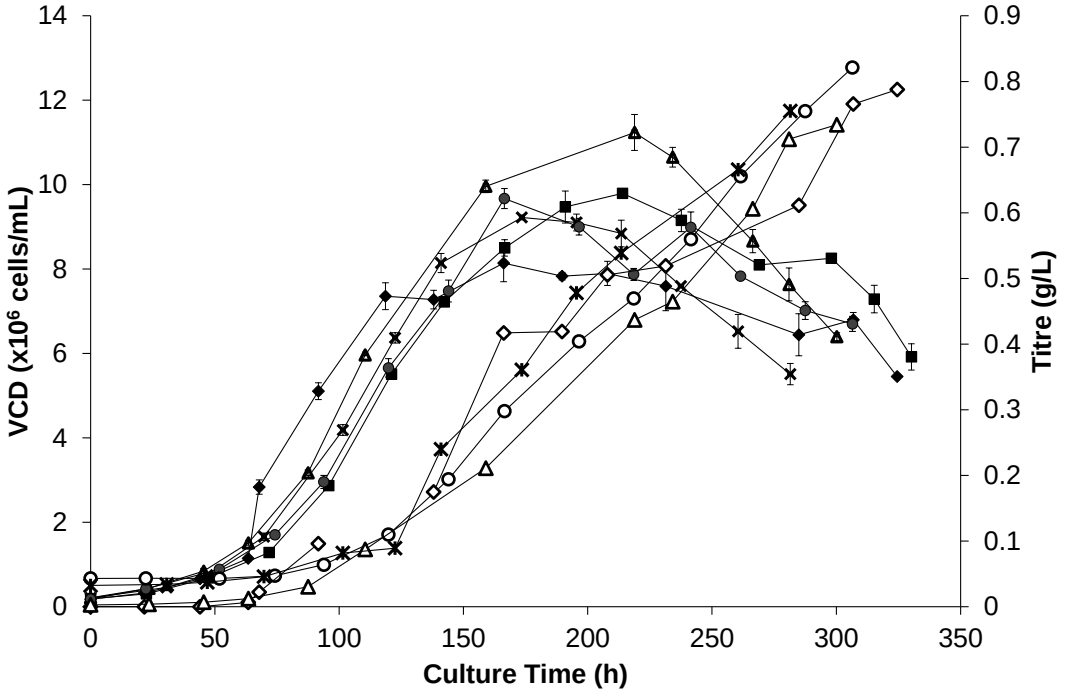

Figure 10.1: *Viable cell concentration (VCD) and titre curves achieved using the 5 L STR described in section 4.3.1.VCD for fed batch run 1(*◆), fed batch run 2 (■), fed batch run 3 (▲), fed batch 4 (X), fed batch run 5 (●). IgG4 titre achieved during the course of the respective batches are shown for fed batch 1 (◇), fed batch 3 (△), fed batch 4 (*), fed batch 5 (○).

10.3 Appendix C

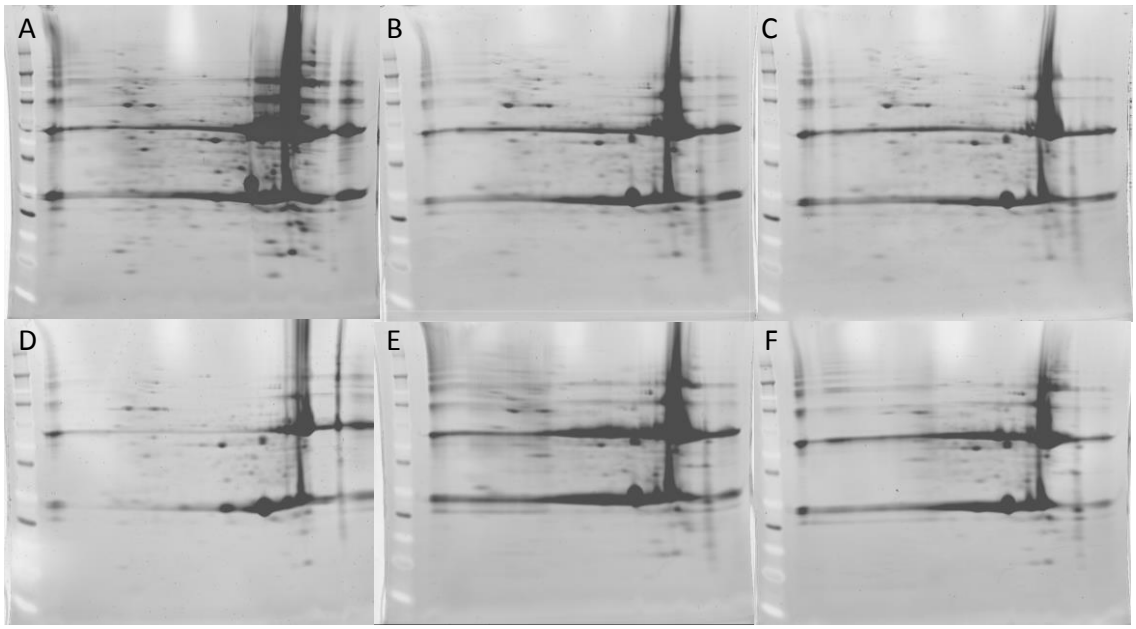

Figure 10.2: *Example 2D PAGE images used for image analysis and comparison of HCP removal across the primary recovery technologies tested. The images shown include the cell culture test material used to test the primary recovery technologies (A); centrifugation and 05 SP depth filtration medium option (B); centrifugation and 10 SP depth filtration medium option (C); centrifugation and 30ZA depth filtration option (D); Bio-Optimal MF-SL™ option (E); QSD™ option (F).*

10.4 Appendix D

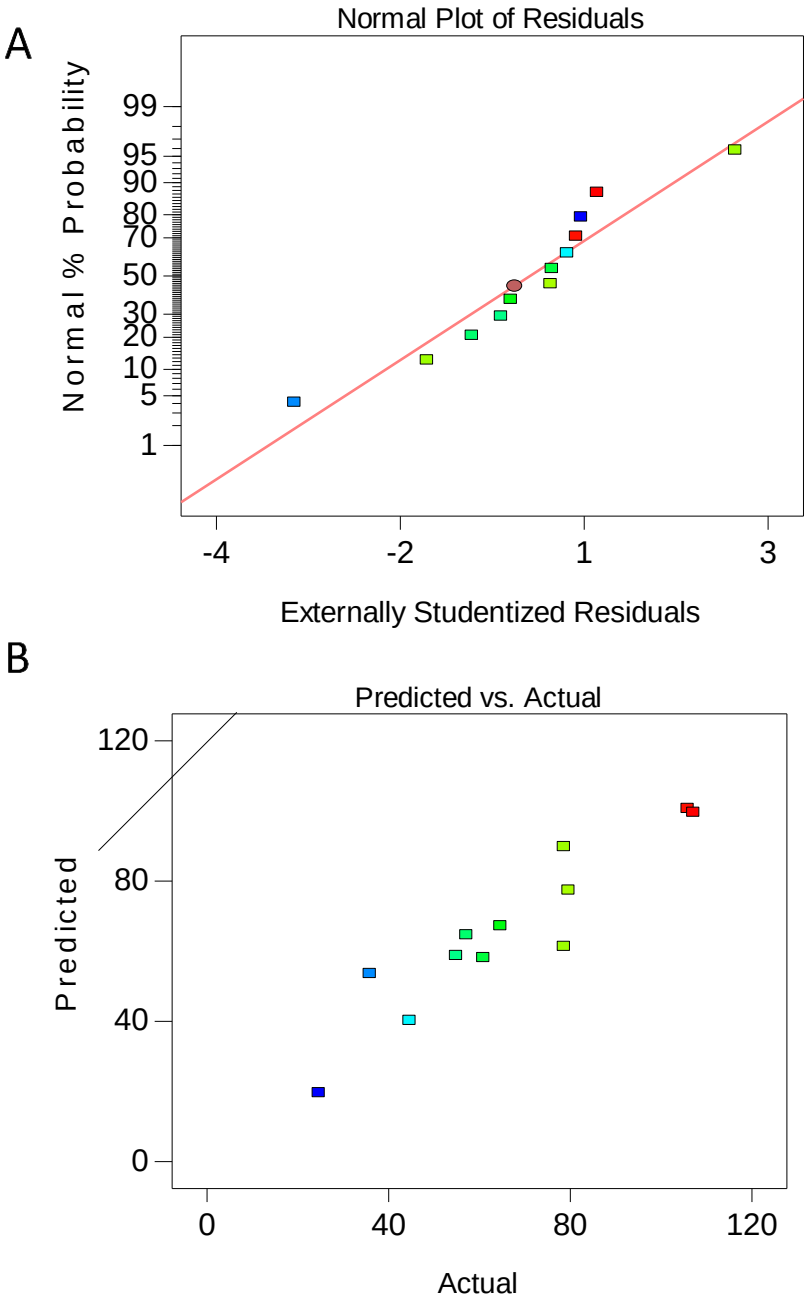

Figure 10.3: *Statistical diagnostic charts for the throughput response of Bio-Optimal MF-SL™ under the selected model conditions.* **A:** *Normal plot of studentised residuals.* **B:** *A plot of throughput predicted by the selected model versus the actual experimental results.*

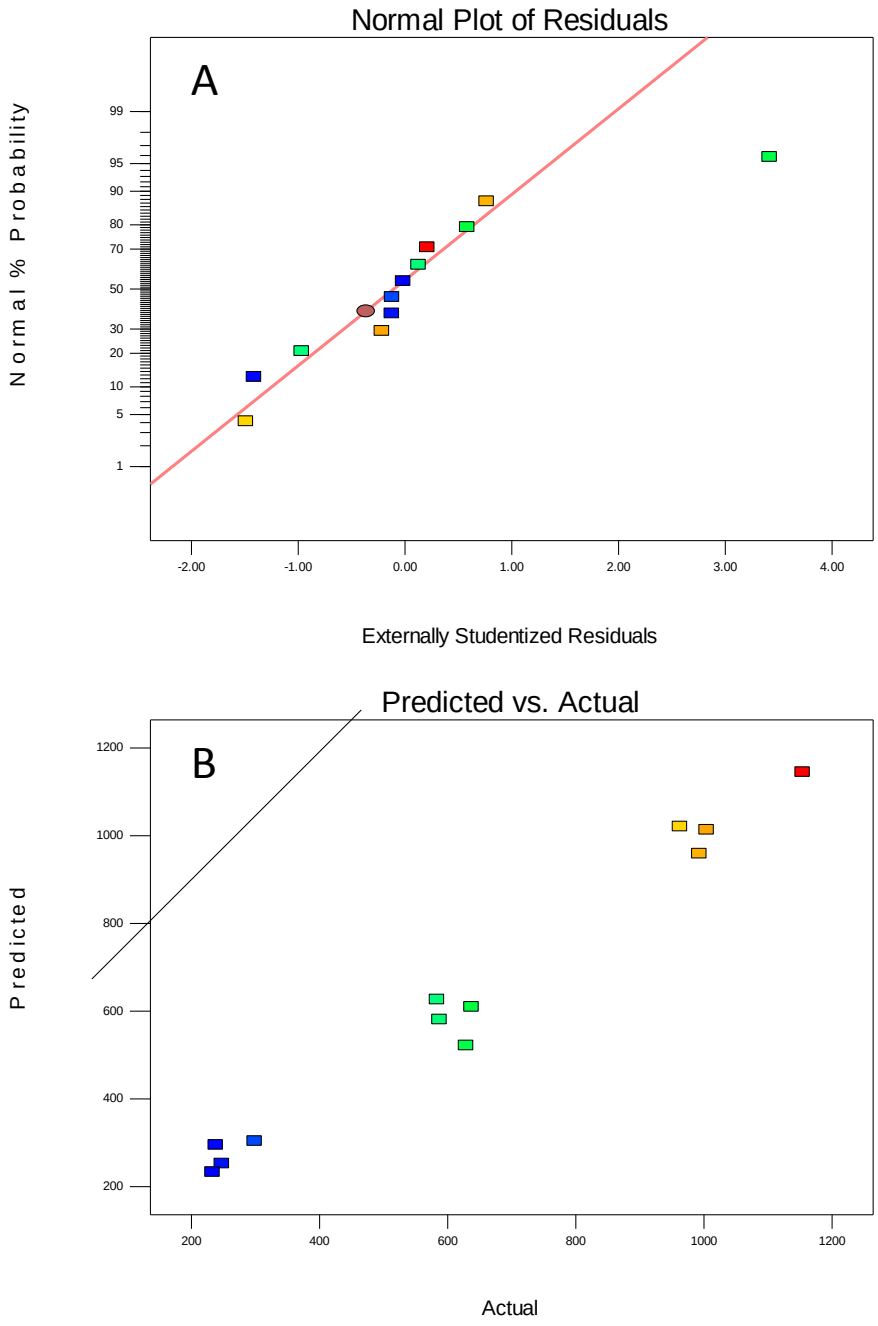

Figure 10.4: *Statistical diagnostic charts for the IgG_1 concentration in the permeate response of Bio-Optimal MF-SL™ under the selected model conditions.* **A:** *Normal plot of studentised residuals.* **B:** *A plot of IgG_1 concentration in the permeate predicted by the selected model versus the actual experimental results.*

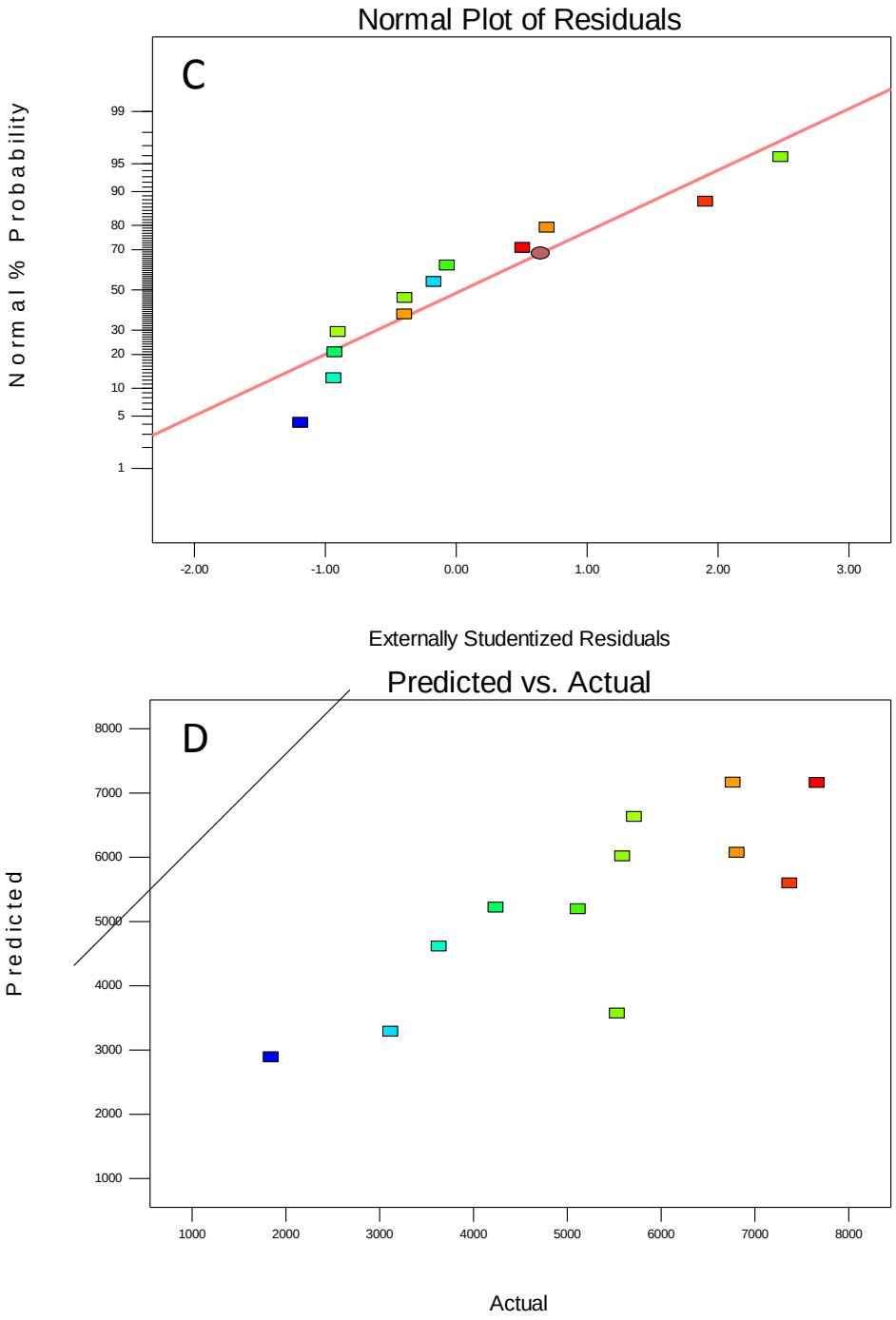

Figure 10.5: *Statistical diagnostic charts for the HCP concentration in the permeate response of Bio-Optimal MF-SL™ under the selected model conditions.* **A:** *Normal plot of studentised residuals.* **B:** *A plot of HCP concentration in the permeate predicted by the selected model versus the actual experimental results.*

10.5 Appendix E

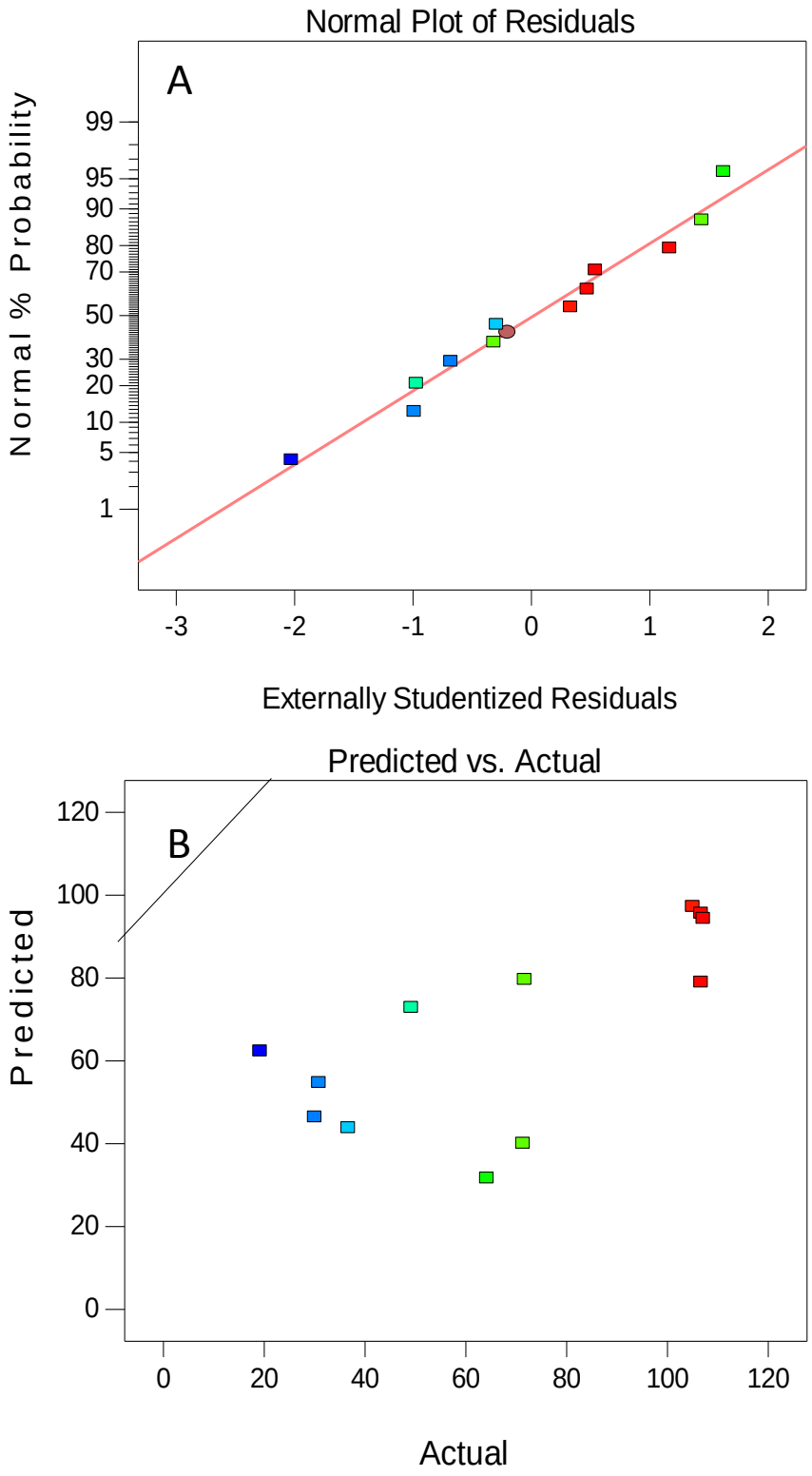

Figure 10.6: *Statistical diagnostic charts for the throughput response of QSD™ under the selected model conditions.* **A:** *Normal plot of studentised residuals.* **B:** *A plot of throughput predicted by the selected model versus the actual experimental results.*

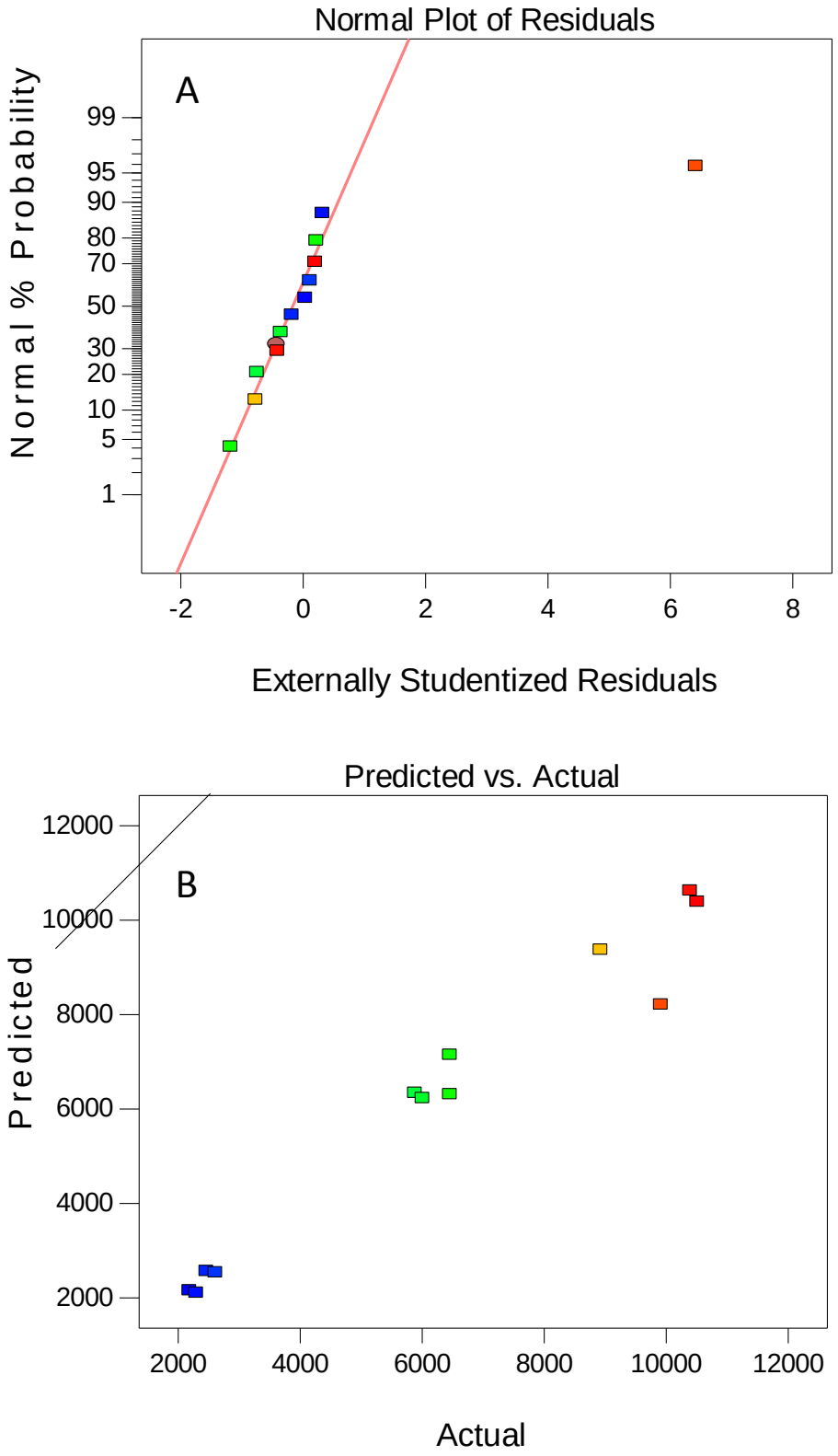

Figure 10.7: *Statistical diagnostic charts for the IgG_1 concentration in the permeate response of QSD™ under the selected model conditions. A: Normal plot of studentised residuals. B: A plot of IgG_1 concentration in the permeate predicted by the selected model versus the actual experimental results*

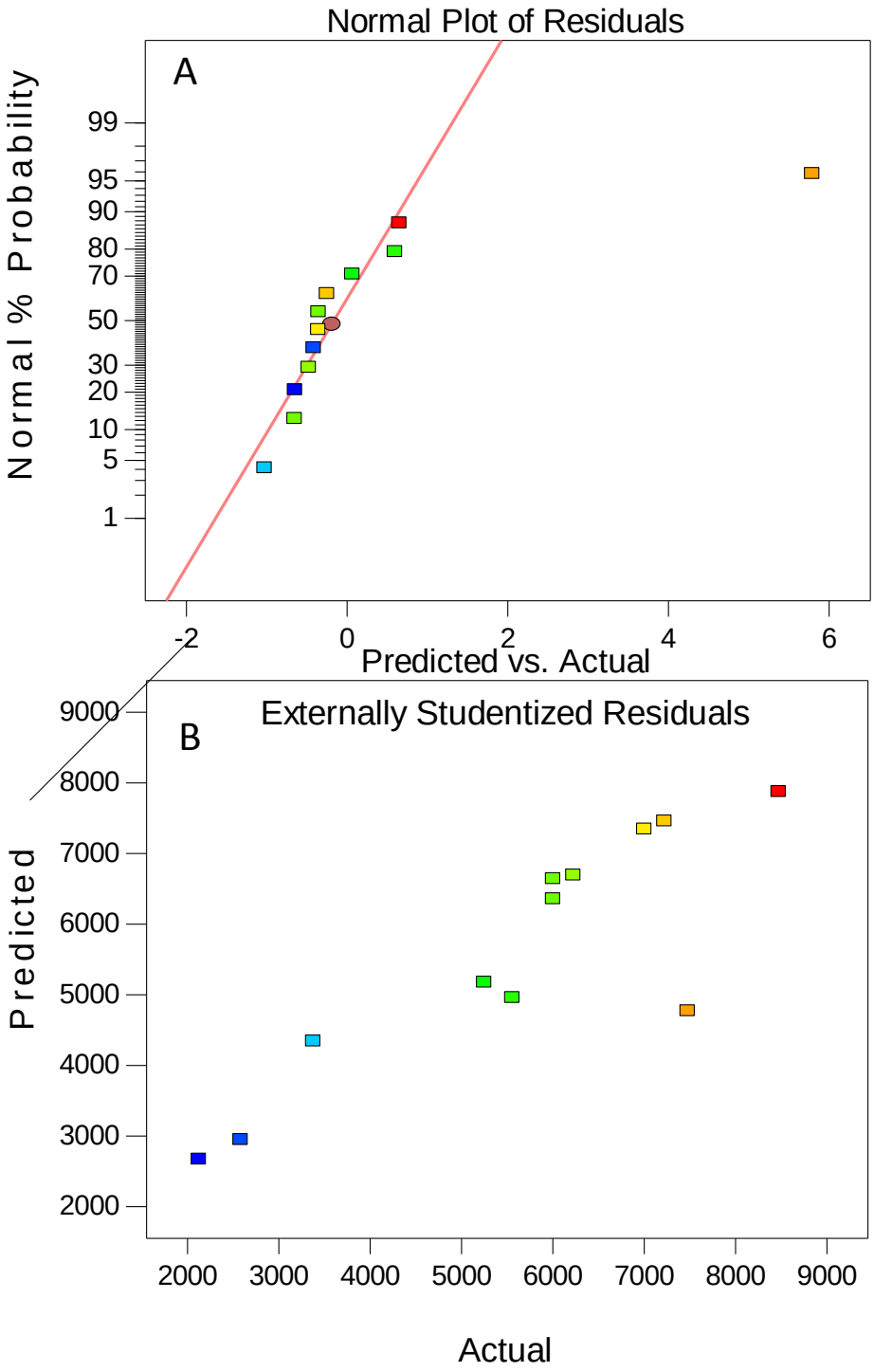

Figure 10.8: *Statistical diagnostic charts for the HCP concentration in the permeate response of QSD™ under the selected model conditions. A: Normal plot of studentised residuals. B: A plot of HCP concentration in the permeate predicted by the selected model versus the actual experimental results.*