

High Density CHO cell cultures:

Improved productivity and product quality



A thesis submitted to DCU for the degree of Doctor of Philosophy (Ph.D)

by

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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List of Figures	X
List of Tables	XX
Abstract	XXIV

Chapter 1: Thesis Introduction: High Density CHO Cell Cultures: Improved Productivity and Product Quality

<i>1.1: Emergence of mammalian cells for recombinant protein production</i>	3
<i>1.2: Factors effecting high cell density growth and productivity of cells</i>	6
1.2.1: Temperature	6
1.2.2: pH	8
1.2.3: Osmolality and concentration of dissolved CO ₂	9
1.2.4: Concentration of dissolved oxygen	10
1.2.5: Concentration of essential nutrients and metabolic by-products	14
1.2.6: Feeding strategy	17
1.2.7: Immobilisation of mammalian cells for enhanced cell densities and higher productivities	22
<i>1.3: Quality of produced recombinant proteins</i>	26
1.3.1: N-glycan biosynthesis	27
1.3.1.1: N-glycan trimming in the ER	29
1.3.1.2: N-glycan processing in the golgi	29
1.3.1.3: O-linked glycans	31
1.3.2: Biological properties of glycosylation	32
1.3.3: Aggregation and glycosylation	34
1.3.3.1: Chemical instabilities	35
1.3.3.2: Physical instabilities	36
1.3.3.3: Protein aggregation	36
1.3.3.4: Aggregation problems at upstream level	38
1.3.4: Culture conditions effecting glycosylation	39
1.3.4.1: Host cell and protein structure	40
1.3.4.2: Process conditions	42
1.3.4.2.1: Physical parameters	42

1.3.4.2.2: Chemical parameters	47
1.4: Glycosylation of IgG1	52
1.5: Objectives of the PhD thesis	54
Chapter 2: Material and Methods	
2.1: Cell line and inoculums development	60
2.2: Cell cultures	61
2.2.1: Cultivation of CHO DP-12 cells in varying concentrations of L-GLN, L-GLU and Ammonia	61
2.2.1.1: Culture medium	61
2.2.1.2: Preparation of Insulin and MTX stock solutions	62
2.2.2: Cultivation of CHO DP-12 cells in suspension in bench top minifors reactor under batch cultivation conditions	62
2.2.2.1: Estimation of maximum shear stress under stirred conditions in 1.7 L minifors reactor	63
2.2.3: Cultivation of CHO DP-12 cells in Alginate-poly-L-lysine-alginate microcapsules in bench top minifors reactor and 1 L Erlenmeyer shake flasks under batch and control-fed perfusion cultivation conditions	65
2.2.3.1: Alginate-poly-L-lysine-alginate microcapsule formation	65
2.2.3.2: CHO DP-12 cell encapsulation in Alginate-poly-L-lysine- alginate microcapsules	67
2.2.3.3: Bioreactor and shake flask conditions for encapsulated cultures	69
2.2.3.4: Control-fed perfusion strategy	69
2.3: Sample analysis	70
2.3.1: Biomass determination in suspension cultures	70
2.3.2: Biomass determination in encapsulated cultures	70
2.3.3: Metabolite determination	71
2.3.4: Capsule size determination	72
2.3.5: Mechanical strength of microcapsules using a texture analyser	72
2.3.6: Determination of capsule permeability to rIgG1 protein	74
2.3.7: IgG1 purification and quantification	75
2.3.8: IgG1 N-glycan analysis	75

2.3.8.1: Glycan release and labelling	75
2.3.8.2: HILIC fluorescence	76
2.3.8.3: Assignment of GU values	77
2.3.8.4: Glycan nomenclature	79
2.3.9: IgG1 aggregation analysis	80
2.4: Calculations	81

Chapter 3: Determination of the effect of Ammonia, L-glutamine and L-glutamine substitutes on rIgG1 glycosylation and aggregation

3.1: Introduction	84
3.2: Results	89
3.2.1: Effect on CHO cell growth and viability	89
3.2.2: Effect on cell metabolism	93
3.2.3: Effect on rIgG1 production	101
3.2.4: Effect on rIgG1 quality	103
3.3: Discussion	113
3.3.1: Effect of L-GLN boosting	114
3.3.2: Substitution of L-GLN with L-GLU	121
3.3.3: Influence of high initial ammonia concentrations	124
3.4: Conclusion	131

Chapter 4: Scale of CHO DP-12 cultures from shake flask to bench-scale minifors reactor

4.1: Introduction	134
4.2: Results	137
4.2.1: Effect on CHO cell growth and viability	137
4.2.2: Effect on cell metabolism	141
4.2.3: Effect on rIgG1 production	145
4.2.4: Effect on rIgG1 quality	149
4.2.5: Estimation of maximum shear stress under stirred conditions	155

4.3: Discussion	156
4.4: Conclusion	161

Chapter 5: Encapsulation of CHO-DP12 cells in Alginate-Poly-L-lysine-Alginate microcapsules: Implications on rIgG1 quality

5.1: Introduction	165
5.2: Results	168
5.2.1: Characterisation of microcapsules	169
5.2.1.1: Capsule size	169
5.2.1.2: Capsule strength	170
5.2.1.3: Permeability of microcapsules	172
5.2.2: Effect of encapsulation on CHO cell growth	173
5.2.2.1: Growth of encapsulated cells	173
5.2.2.2: Growth of cells in capsules –v- suspension	175
5.2.3: Effect of encapsulation on rIgG1 production	178
5.2.4: Effect of encapsulation on cell metabolism	181
5.2.5: Effect of encapsulation on product quality	185
5.3: Discussion	188
5.4: Conclusion	198

Chapter 6: Application of a control-fed perfusion strategy for enhanced colonisation of Alginate-poly-L-lysine-alginate with CHO DP-12 cells; Implications on rIgG1 quality

6.1: Introduction	201
6.2: Results	207
6.2.1: Batch encapsulated –v- Perfusion encapsulated in 1.7 L minifors reactor	209
6.2.1.1: Capsule characterisation	209
6.2.1.1.1: Capsule size	209
6.2.1.1.2: Capsule strength	211

6.2.1.2: Growth of encapsulated cells under batch and perfusion modes of Cultivation	212
6.2.1.2.1: Growth of cells inside microcapsules	212
6.2.1.2.2: Viable cell numbers per ml _{reactor}	216
6.2.1.3: Effect of perfusion mode on CHO cell productivity	220
6.2.1.4: Effect of perfusion mode on CHO cell metabolism	222
6.2.1.5: Effect of perfusion mode on product quality	227
6.2.2: Batch encapsulated –v- perfusion encapsulated in 1 L Erlenmeyer shake flasks	231
6.2.2.1: Capsule characterisation	232
6.2.2.1.1: Capsule size	232
6.2.2.1.2: Capsule strength	233
6.2.2.2: Comparability of encapsulated cell growth under batch conditions in 1 L Erlenmeyer shake flasks and 1.7 L minifors reactors	235
6.2.2.3: Comparability of encapsulated cell metabolism under batch conditions in 1 L Erlenmeyer shake flasks and 1.7 L minifors reactors	240
6.2.2.4: Comparability of encapsulated cell productivity under batch conditions in 1 L Erlenmeyer shake flasks and 1.7 L minifors reactors	243
6.2.2.5: Comparability of encapsulated cell product quality under batch conditions in 1 L Erlenmeyer shake flasks and 1.7 L minifors reactors	245
6.2.3: Cultivation of encapsulated cells in shake flasks under batch (non-CO ₂ incubator), perfusion (non-CO ₂ incubator) and perfusion (CO ₂ incubator) cultivation conditions	252
6.2.3.1: Capsule characterisation	253
6.2.3.1.1: Capsule size	253
6.2.3.1.2: Capsule strength	254
6.2.3.1.3: Capsule permeability	255
6.2.3.2: Growth of encapsulated cells in 1L Erlenmeyer shake flasks under conditions of batch (non-CO ₂ incubator), perfusion (non-CO ₂ incubator) and perfusion (CO ₂ incubator) mode of cultivation.	259
6.2.3.3: Metabolic analysis of encapsulated cells cultured in 1L	

Erlenmeyer shake flasks under conditions of batch (non-CO ₂ incubator), controlled-fed perfusion (non-CO ₂ incubator and CO ₂ incubator) modes of cultivation.	266
6.2.3.4: Productivity analysis of encapsulated cells cultured in 1L Erlenmeyer shake flasks under conditions of batch (non-CO ₂ incubator), controlled-fed perfusion (non-CO ₂ incubator and CO ₂ incubator) modes of cultivation.	275
6.2.3.5: Product quality analysis of encapsulated cells cultured in 1L Erlenmeyer shake flasks under conditions of batch (non-CO ₂ incubator), controlled-fed perfusion (non-CO ₂ incubator and CO ₂ incubator) modes of cultivation.	279
6.2.3.5.1: Batch encapsulated culture -v- Controlled-fed perfusion (non-CO ₂ incubator) encapsulated culture	281
6.2.3.5.2: Controlled-fed perfusion (non-CO ₂ incubator) -v- Controlled-fed perfusion (CO ₂ incubator) encapsulated Cultures	285
6.3: Discussion	289
6.4: Conclusion	311
Chapter 7: Overall Thesis Conclusions and Outlooks	314
Bibliography	323
Appendices	352
Appendix A	352
Appendix B	357

List of Figures

Figure 1.2.5.1: Overview of the metabolic fates of glucose and glutamine in mammalian cells (Teixeira et al., 2005).....	16
Figure: 1.3.1.1: Core N-glycan structure (Butler, 2006).....	27
Figure: 1.3.1.2: N-glycan precursor attached to dolichol carrier (Butler, 2006).....	29
Figure 1.3.1.2.1: Glycosylation pathway for N-glycans (Butler, 2006).....	30
Figure 1.3.1.2.2: Production of tri- and tetra-antennary structures.....	31
Figure 1.3.1.3.1: Processing of O-linked glycans in CHO cells (Butler, 2006).....	32
Figure 1.4.1: IgG1 structure containing one consensus N-linked glycosylation site as Asn297	54
Figure 2.2.3.1: Schematic representation of the Inotech Encapsulator® according to (Gugerli, 2003).....	67
Figure 2.3.5.1: Schematic compression of a capsule by Texture Analyser piston (Gugerli, 2003).....	73
Figure 3.2.1.1: Correlation of the viable cell density displayed by cells over time when cultured in varying concentrations of L-GLN, L-GLU and ammonia	91
Figure 3.2.1.2: Correlation of the % viability over the cultivation period for cells grown in the presence of varying concentrations of GLN, GLU and Amm.....	92
Figure 3.2.2.1: Concentration of glucose present in cultures containing varying concentrations of L-GLN, L-GLU and ammonia over the duration of the respective cultivation periods.....	96
Figure 3.2.2.2: Concentration of lactate present in cultures containing varying concentrations of L-GLN, L-GLU and ammonia over the duration of the respective cultivation periods.....	97
Figure 3.2.2.3: Concentration of glutamine present in cultures containing varying concentrations of L-GLN, L-GLU and ammonia over the duration of the respective cultivation periods.....	98

Figure 3.2.2.4: Concentration of ammonia present in cultures containing varying concentrations of L-GLN, L-GLU and ammonia at the end of the exponential and stationary culture periods.....	99
Figure 3.2.3.1: Concentration of rIgG1 purified from cell free media samples at the end of the exponential and stationary growth phases of cells cultured in varying concentrations of L-GLN, L-GLU and ammonia.....	102
Figure 3.2.4.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from cultures containing varying concentrations of L-GLN, L-GLU and Amm at the end of the exponential growth phase.....	104
Figure 3.2.4.2: Relative % of F(6)A1/A2 glycan forms detected on rIgG1 samples harvested as detected using HILIC following PNGaseF release and 2-AB labelling.....	106
Figure 3.2.4.3: Relative % of M5 glycan forms detected on rIgG1 samples harvested as detected using HILIC following PNGaseF release and 2-AB labelling.....	107
Figure 3.2.4.4: Relative % of F(6)A2[6]G(4)1 glycan forms detected on rIgG1 samples harvested as detected using HILIC following PNGaseF release and 2-AB labelling.....	108
Figure 3.2.4.5: Relative % of F(6)A2[3]G(4)1 glycan forms detected on rIgG1 samples harvested as detected using HILIC following PNGaseF release and 2-AB labelling.....	109
Figure 3.2.4.6: Relative % of F(6)A2G2 glycan forms detected on rIgG1 samples harvested as detected using HILIC following PNGaseF release and 2-AB labelling.....	110
Figure 3.2.4.7: Relative % of A2G2 glycan forms detected on rIgG1 samples harvested as detected using HILIC following PNGaseF release and 2-AB labelling.....	111
Figure 3.2.4.8: Relative % of F(6)A2G2S1 glycan forms detected on rIgG1 samples harvested as detected using HILIC following PNGaseF	

release and 2-AB labelling.....	112
Figure 3.2.4.9: Relative % monomeric form of rIgG1 samples as detected by SEC.....	113
Figure 3.3.1.1: Role of glutamine and glucose in nucleotide sugar donor synthesis Nyberg et al., (1999).....	121
Figure 4.2.1.1: Growth of CHO cells cultures in 1L Erlenmeyer shake flasks and 1.7 L minifors reactor.....	139
Figure 4.2.1.2: % viability of CHO cells cultures in 1L Erlenmeyer shake flasks and 1.7 L minifors reactor.....	140
Figure 4.2.2.1: Concentration of glutamine and ammonia present in both 1 L Erlenmeyer shake flask cultures (SF) and 1.7 L minifors reactor cultures (MR) over the duration of the respective cultivation periods.....	143
Figure 4.2.2.2: Concentration of glucose and lactate present in both 1 L Erlenmeyer shake flask cultures (SF) and 1.7 L minifors reactor cultures (MR) over the duration of the respective cultivation periods.....	144
Figure 4.2.3.1: Concentration of rIgG1 purified from cell free media samples harvested from both 1 L Erlenmeyer shake flask cultures and 1.7 L minifors reactor cultures.....	147
Figure 4.2.3.2: Concentration of rIgG1 purified from cell free media samples at the end of the stationary growth phases of cells cultured in 1L Erlenmeyer shake flasks and 1.7 L minifors reactors.....	148
Figure 4.2.4.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from 1 L Erlenmeyer shake flask and 1 L minifors reactor studies at the end of the stationary growth phase.....	151
Figure 4.2.4.2: Relative % of F(6)A1/A2 glycan forms detected on rIgG1 samples harvested from 1 L Erlenmeyer shake flask and 1 L minifors reactor studies at the end of the stationary growth phase.....	152
Figure 4.2.4.3: Relative % of F(6)A2[6]G(4)1 glycan forms detected on rIgG1 samples harvested from 1 L Erlenmeyer shake flask and 1 L minifors reactor studies at the end of the stationary growth phase.....	153
Figure 4.2.4.4: Relative % of F(6)A2G2 glycan forms detected on rIgG1 samples	

harvested from 1 L Erlenmeyer shake flask and 1 L minifors reactor studies at the end of the stationary growth phase.....	154
Figure 4.2.4.5: Relative % of rIgG1 harvested from cultures at the end of the stationary phase in monomeric form analysed used SEC.....	155
Figure 5.2.1.1.1: Capsule radius size (μm) over the duration of the cultivation period for each of the three encapsulated cultures completed.....	170
Figure 5.2.1.2.1: Mechanical resistance (force (g)/capsule) over cultivation period for 300 μm APA microcapsules containing CHO DP-12 cells cultivated in a 1.7 L minifors reactor.....	172
Figure 5.2.1.3.1: Confocal microscopy analysis (100 X) of 300 μm APA microcapsules incubated in fluorescent dextran (150-50kDa).....	173
Figure 5.2.2.1.1: Microscopic analysis (40 X) of microcapsule colonisation by cells over the cultivation days 0, 2, 4 and 6.....	174
Figure 5.2.2.1.2: Microscopic analysis (400 x and 600 x) of the cluster of cells present inside microcapsules on day 6 of the culture period.....	175
Figure 5.2.2.2.1: Growth of CHO cells cultures in suspension and encapsulated 300 μm liquid core APA microcapsules.....	176
Figure 5.2.2.2.2: % Viability of CHO cells cultured in suspension and encapsulated in 300 μm liquid capsules.....	177
Figure 5.2.3.1: Concentration of rIgG1 purified from cell free media samples harvested from encapsulated cultures on day 5 onwards and suspension cultures on day 4 onwards.....	179
Figure 5.2.3.2: Concentration of rIgG1 purified from cell free media samples at the end of the exponential and stationary growth phases of cells cultured in suspension and in 300 μm radius liquid capsules.....	180
Figure 5.2.4.1: Concentration of glucose and lactate present in both suspension cultures (SC) and encapsulated cultures (EC) over the duration of the respective cultivation periods.....	183
Figure 5.2.4.2: Concentration of glutamine and ammonia present in both suspension	

cultures (SC) and encapsulated cultures (EC) over the duration of the respective cultivation periods.....	184
Figure 5.2.5.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from 1 L encapsulated and suspension cultures at the end of the stationary growth phase. N-glycans were released from the rIgG1 samples.....	187
Figure 5.2.5.2: Relative % monomeric form of rIgG1 samples as detected by SEC.....	188
Figure 5.3.1: Confocal analysis of the surface APA microcapsules containing cells sampled on day 1 from culture.....	195
Figure 6.2.1.1.1: Average capsule radius size (μm) +/- STD over the duration of the cultivation period for each of the three batch encapsulated cultures completed and the control-fed perfusion culture.....	210
Figure 6.2.1.1.2.1: Mechanical resistance (force (g)/capsule) over cultivation period for 300 μm APA microcapsules containing CHO DP-12 cells cultivated in a 1.7 L minifors reactor under batch and control-fed perfusion conditions.....	212
Figure 6.2.1.2.1.1: Microscopic analysis (40 X) of microcapsule colonisation by cells on cultivation days 6 and 8 in both batch and perfusion cultures.....	214
Figure 6.2.1.2.1.2: Growth of CHO cells cultures in 300 μm liquid core APA microcapsules under batch and perfusion modes of cultivation.....	215
Figure 6.2.1.2.2.1: Growth of encapsulated CHO cells cultures 300 μm liquid core APA microcapsules in a 1.7 L minifors reactor under batch and perfusion modes of cultivation.....	218
Figure 6.2.1.2.2.2: % Viability of CHO cells encapsulated in 300 μm liquid capsules and cultured under batch and perfusion modes of cultivation.....	219
Figure 6.2.1.3.1: Total amount of IgG1 (μg) purified from cell free media samples harvested from encapsulated cultures on day 5 onwards.....	221
Figure 6.2.1.4.1: Concentration of glucose and lactate present in both batch and perfusion cultures over the duration of the respective cultivation periods.....	224

Figure 6.2.1.4.2 Concentration of glutamine and ammonia present in both batch and perfusion cultures over the duration of the respective cultivation period.....	225
Figure 6.2.1.5.1 Relative % of N-glycan forms detected on rIgG1 samples harvested from batch and perfusion encapsulated cultures on day 5 of the culture period (before perfusion was initiated) N-glycans were released from the rIgG1.....	228
Figure 6.2.1.5.2: Relative % of M5 present on rIgG1 harvested from batch encapsulated cultures and controlled perfusion cultures at the end of the stationary growth phases (day 7 batch and day 8 controlled-fed perfusion).....	229
Figure 6.2.1.6.3: Relative % monomeric form of rIgG1 samples as detected by SEC.....	231
Figure 6.2.2.1.1: Average capsule radius size (μm) +/- STD over the duration of the cultivation period for each of the three encapsulated cultures completed in both 1.7 L minifors reactor and 1 L Erlenmeyer shake flask platforms.....	233
Figure 6.2.2.1.2.1: Mechanical resistance (force (g)/capsule) over cultivation period for 300 μm APA microcapsules containing CHO DP-12 cells cultivated in a 1.7 L minifors reactor and 1 L Erlenmeyer shake flask platforms under batch cultivation conditions.....	234
Figure 6.2.2.2.1 Growth of CHO cells cultures in 300 μm liquid core APA microcapsules under batch cultivation conditions in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms.....	237
Figure 6.2.2.2.2: Growth of CHO DP-12 cells both encapsulated in 300 μm APA microcapsules and freely in suspension under batch modes of cultivation in 1L Erlenmeyer shake flask and 1.7 L minifors reactor platforms.....	238
Figure 6.2.2.2.3: % Viability of CHO cells cultured in suspension and encapsulated in 300 μm liquid capsules in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor cultures.....	239
Figure 6.2.2.4.1: Concentration of rIgG1 purified from cell free media samples	

harvested from encapsulated and suspension cultures conducted in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor cultures.....	244
Figure 6.2.2.5.1: Relative % of F6A2 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flasks at the end of the exponential and stationary growth phases.....	246
Figure 6.2.2.5.2: Relative % of M5 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flasks at the end of the exponential and stationary growth phases.....	247
Figure 6.2.2.5.3: Relative % of F(6)A2[6]G(4)1 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flasks at the end of the exponential and stationary growth phases.....	247
Figure 6.2.2.5.4: Relative % of F(6)A2G2 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flasks at the end of the exponential and stationary growth phases.....	248
Figure 6.2.2.5.5: Relative % of A2G2 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flasks at the end of the exponential and stationary growth phases.....	248
Figure 6.2.2.5.6: Relative % of N-glycan forms detected on rIgG1 samples harvested from batch suspension and encapsulated cultures performed in both 1 L Erlenmeyer shake flask and 1.7 L minifors reactor models.....	249
Figure 6.2.2.5.7: Relative % of M5 and F(6)A2G2S1 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor cultures at the end of the stationary growth phase.....	250
Figure 6.2.2.5.8: Relative % of F(6)A2G2S1 present on rIgG1 harvested from batch encapsulated and suspension cultures in 1 L Erlenmeyer shake flask at the end of the stationary growth phase.....	251
Figure 6.2.2.5.9: Relative % monomeric from of rIgG1 samples as detected by SEC.....	251
Figure 6.2.3.1.1.1: Average capsule radius size (μm) +/- STD over the duration of the cultivation period for each of the three encapsulated cultures completed and capsule radius size over the duration of	

the single perfusion culture.....	253
Figure 6.2.3.1.2.1: Mechanical resistance (force (g)/capsule) over cultivation period for 300 μm APA microcapsules containing CHO DP-12 cells cultivated under batch and control-fed perfusion conditions in 1 L Erlenmeyer shake flasks.....	255
Figure 6.2.3.1.3.1: Permeability of 300 μm APA microcapsules to dextrans of varying molecular weight during a batch culture in 1 L Erlenmeyer shake flasks in a non-CO ₂ incubator.....	256
Figure 6.2.3.1.3.2: Permeability of 300 μm APA microcapsules to dextrans of varying molecular weight during a control-fed perfusion culture in 1 L Erlenmeyer shake flasks in a non-CO ₂ incubator.....	257
Figure 6.2.3.1.3.3: Permeability of 300 μm APA microcapsules to dextrans of varying molecular weight during a control-fed perfusion culture in 1 L Erlenmeyer shake flasks in a 5 % CO ₂ incubator.....	258
Figure 6.2.3.2.1: Microscopic analysis of microcapsule colonisation by cells cultured under batch and controlled-fed perfusion modes of cultivation in both non-CO ₂ and CO ₂ incubators.....	261
Figure 6.2.3.2.2: Growth of CHO cells cultures in 300 μm liquid core APA microcapsules under batch and controlled-fed perfusion (non-CO ₂ and CO ₂ incubators) cultivation conditions in 1 L Erlenmeyer shake flask..	262
Figure 6.2.3.2.3: LN plot of viable cell densities (cells/ml _{vessel}) over the cultivation period for cells encapsulated in 300 μm APA microcapsules and cultured under batch and controlled-fed perfusion (non-CO ₂ and CO ₂ incubator) modes of cultivation in 1 L Erlenmeyer shake flasks.....	263
Figure 6.2.3.2.4: Growth of CHO cells cultures in 300 μm liquid core APA microcapsules under batch and controlled-fed perfusion (non-CO ₂ and CO ₂ incubators) cultivation conditions in 1 L Erlenmeyer shake flask..	264
Figure 6.2.3.2.5: % Viability of CHO cells cultured encapsulated in 300 μm liquid capsules and cultured in 1 L Erlenmeyer shake flask under batch	

and controlled-fed perfusion (non-CO ₂ and CO ₂ incubator) modes of cultivation.....	265
Figure 6.2.3.3.1: Concentration of glucose present in batch and controlled-fed perfusion cultures over the duration of the respective cultivation period.....	270
Figure 6.2.3.3.2: Concentration of lactate present in batch and controlled-fed perfusion cultures over the duration of the respective cultivation period.....	271
Figure 6.2.3.3.3: Concentration of glutamine present in batch and controlled-fed perfusion cultures over the duration of the respective cultivation period.....	272
Figure 6.2.3.3.3: Concentration of ammonia present in batch and controlled-fed perfusion cultures over the duration of the respective cultivation period.....	273
Figure 6.2.3.4.1: Concentration of rIgG1 present in encapsulated batch and controlled-fed perfusion (non-CO ₂ and CO ₂ incubator) 1 L Erlenmeyer shake flask cultures as quantified over the course of the culture period.....	277
Figure 6.2.3.4.2: Total amount of IgG1 (µg) purified from cell free media samples harvested from batch and control-fed perfusion encapsulated cultures on alternating days.....	280
Figure 6.2.3.5.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from encapsulated cultures on day 5 conducted in 1 L Erlenmeyer shake flask cultures under batch and control-fed perfusion modes of cultivation..	282
Figure 6.2.3.5.2: Relative % monomeric form of rIgG1 samples as detected by SEC.....	281
Figure 6.3.3.5.1.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from encapsulated cultures conducted in 1 L Erlenmeyer shake flask cultures under batch and control-fed perfusion modes of cultivation. Samples were harvested at the end of the stationary growth period.....	283
Figure 6.2.3.5.2.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from encapsulated cultures conducted in 1 L Erlenmeyer shake flask cultures under control-fed perfusion modes of cultivation in both a non-CO ₂ and 5 % CO ₂ environment. Samples were harvested at the end of the extended growth periods, day 8 for non-CO ₂ incubated cultures	

and day 9 for CO₂ incubated cultures.....289

Figure 6.2.3.5.2.2: Relative % of N-glycan forms detected on rIgG1 samples harvested from encapsulated cultures conducted in 1 L Erlenmeyer shake flask cultures under control-fed perfusion modes of cultivation in both a non-CO₂ and 5 % CO₂ environment. Samples were harvested at the end of the stationary growth periods, day 11 for non-CO₂ incubated cultures and day 13 for CO₂ incubated cultures.....288

List of Tables:

Table 2.2.2.1: 1.7 L minifors reactor characteristics and culture medium properties.....	63
Table 2.3.8.3.1: Assignment of corresponding number and linkage of the component monosaccharide units represented by each GU value elucidated by carrying out a series of exoglycosidase digestions on one of the released glycans.....	78
Table 3.2.1.1: Calculated maximum specific growth rates displayed by cells cultured in varying concentrations of L-GLN, L-GLU and ammonia.....	92
Table 3.2.1.2: Time point (day) which each corresponding growth phase ended in L-GLN, L-GLU and ammonia cultures.....	93
Table: 3.2.2.1: Calculated rates of consumption and production of glucose and lactate as determined by offline analysis throughout the culture period for cells cultured in varying concentrations of L-GLN, L-GLU and ammonia.....	100
Table: 3.2.2.2: Calculated rates of consumption and production of glutamine and ammonia as determined by offline analysis throughout the culture period for cells cultured in varying concentrations of L-GLN, L-GLU and ammonia.....	100
Table 3.2.2.3: Calculated yield of by-product on metabolites over the culture period for each of the culture conditions tested.....	101
Table 3.2.3.1: Calculated rIgG1 production rates and cell specific production rates for cells cultured in varying concentrations of L-GLN, L-GLU and ammonia.....	103
Table 4.2.1.1: Calculated maximum specific growth rates displayed by cells cultured in 1 L Erlenmeyer shake flasks and 1.7 L minifors reactor over the exponential growth period.....	140
Table: 4.2.2.1: Calculated rates of consumption and production of key metabolites as determined by offline analysis throughout the culture period.....	145
Table 4.2.3.1: Calculated rIgG1 production rates and cell specific production rates for cells cultured in 1L Erlenmeyer shake flask and 1.7 L minifors reactors.....	148

Table 4.2.5: Maximum shear stress exerted on the cells under conditions of mechanical agitation and aeration in 1.7 L minifors reactor.....	155
Table 5.2.2.2.1: Calculated maximum specific growth rates displayed by cells cultured in suspension and encapsulated in 300 µm liquid capsules over the exponential growth period.....	177
Table 5.2.3.1: Calculated rIgG1 production rates and cell specific production rates for cells cultured in suspension and encapsulated in 300 µm radius liquid capsules.....	180
Table: 5.2.4.1: Calculated rates of consumption and production of key metabolites as determined by offline analysis throughout the culture period.....	185
Table 6.2.1.2.1.1: Calculated maximum viable cell densities with respect to per ml capsule for cells cultured under batch and perfusion modes of cultivation in a 1.7 L minifors reactor culture.....	216
Table 6.2.1.2.2.1: Calculated maximum specific growth rates displayed by cells encapsulated in 300 µm liquid capsules over the exponential growth period.....	219
Table 6.2.1.3.1: Calculated rIgG1 production rates and cell specific production rates for cells encapsulated in 300 µm radius liquid capsules and cultured under batch and perfusion modes of cultivation.....	221
Table 6.3.1.4.1: Calculated rates of consumption and production of key metabolites (glucose and lactate) as determined by offline analysis throughout the culture period.....	226
Table 6.3.1.4.2: Calculated rates of consumption and production of key metabolites (glutamine and ammonia) as determined by offline analysis throughout the culture period.....	226
Table 6.2.1.5.1: Relative % of M5 present on rIgG1 harvested from batch and control-fed perfusion cultures on days 5-7 as detected using HILIC following PNGaseF release and 2-AB labelling.....	229
Table 6.2.1.5.2: Relative % of F(6)A2G2S1 present on rIgG1 harvested from batch and control-fed perfusion cultures at the end of the exponential and	

stationary growth phases.....	230
Table 6.2.2.2.1: Calculated maximum specific growth rates displayed by cells cultured in suspension and encapsulated in 300 μm liquid capsules in 1L Erlenmeyer shake flask and 1.7 L minifors reactor platforms over the exponential growth period.....	239
Table 6.2.2.3.1: Calculated volumetric and specific consumption and production rates of glucose and lactate in batch suspension and encapsulated cultures conducted on 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms.....	242
Table 6.2.2.3.2: Calculated volumetric and specific consumption and production rates of glutamine and ammonia in batch suspension and encapsulated cultures conducted on 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms.....	242
Table 6.2.2.3.3: Calculated yields of by-product/metabolite (mmoles/mmoles) over the cultivation period for both encapsulated and suspension cultures conducted using 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms.....	243
Table 6.2.2.4.1: Calculated rIgG1 production rates and cell specific production rates for cells cultured in suspension and encapsulated in 300 μm radius liquid capsules in both 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms.....	245
Table 6.2.3.2.1: Calculated maximum specific growth rates displayed by cells encapsulated in 300 μm liquid capsules and cultures in 1L Erlenmeyer shake flasks under batch and controlled-fed perfusion (non-CO ₂ and CO ₂ incubator) cultivation conditions.....	265
Table 6.2.3.3.1: All glucose and lactate volumetric (mmoles/day) and specific (mmoles/10 ⁶ cells/day) consumption and production kinetics calculated over the exponential phase (days 1-5), before control-fed perfusion strategy was applied to the non-CO ₂ and CO ₂ cultures.....	274
Table 6.2.3.3.2: All glutamine and ammonia volumetric (mmoles/day) and specific (mmoles/10 ⁶ cells/day) consumption and production	

kinetics calculated over the exponential phase (days 1-5), before control-fed perfusion strategy was applied to the non-CO ₂ and CO ₂ cultures.....	274
Table 6.2.3.3.3: All specific glucose, lactate, glutamine and ammonia specific consumption and production rates (mmoles/10 ⁶ cells/ml) over the extended growth period for control-fed perfusion non-CO ₂ (days 5-8) and control-fed perfusion CO ₂ (days 5-9).....	275
Table 6.2.3.3.4: All yields of by-product/metabolite for batch and control-fed perfusion (non-CO ₂ and CO ₂ cultures) over the cultivation period.....	275
Table 6.2.3.4.1: Calculated rIgG1 production rates and cell specific production rates for cells encapsulated in 300 µm radius liquid capsules and cultured under batch and controlled-fed perfusion modes of cultivation in 1 L Erlenmeyer shake flasks.....	278
Table 6.2.3.5.1.1: Overview of the % differences noted in the relative % of glycans detected on rIgG1 harvested from controlled-fed perfusion cultures (non-CO ₂ incubator) at specified time point in the culture period.....	282
Table 6.2.3.5.2.1: Overview of the % differences noted in the relative % of glycans detected on rIgG1 harvested from controlled-fed perfusion cultures (CO ₂ incubator) at specified time point in the culture period.....	285
Table 7.1: Overview of enhancements in maximum viable cell number attained and degree of capsule colonisation over the entire timescale of the project.....	319

Abstract:

“High density CHO cell cultures; improved productivity and product quality”-Karen Byrne

Continual growth in the biopharmaceutical industry in recombinant protein production has resulted in an ever increasing demand for optimised production processes, particularly in the area of upstream mammalian cell culture bioprocessing. The inefficient energy demands of mammalian cells in culture persists as a limiting factor for optimisation of cell culture processes. The Process Analytical Technology (PAT) framework initiative, put forward by the FDA in 2001, outlines measures for the strict monitoring and control of critical process parameters (CPP-s) affecting cell growth, productivity and product quality. The identification of CPP-s affecting protein quality (namely glycosylation and/or aggregation) has never been more important in seeking recombinant protein drug approval. A project was designed aimed at addressing the issue of CPP identification in high density CHO cell cultures producing the recombinant protein IgG1. Initial studies identified the importance of L-glutamine for cell growth and productivity, but also for increased complexity of the glycoform on the IgG1 protein. This was identified by comparing cultures containing 4 mM L-Glutamine to cultures containing 0 and 4 mM L-glutamate. Interestingly this study also identified that the impact of the by-product ammonia on recombinant protein quality may be over-estimated by investigating such effects through the incorporation of high initial ammonia concentrations (~15 mM). In addressing the title of the project, encapsulation as a mode of cultivation for suspension adapted mammalian cells was also investigated, with significantly increased yields in cell (3.7-fold) and product titres being achieved for batch encapsulated cultures in comparison to suspension cultures. Research to date has failed to identify if encapsulation may have implications on protein quality. Initial studies identified that the quality of the protein harvested at the end of the stationary growth period in both batch and suspension cultures was relatively similar. In order to further increase the maximum cell yields and product titres obtained in an encapsulated culture, a control-fed perfusion strategy was designed and applied to the encapsulated cells. Further increased cell yields, 10-fold higher than that which was achieved in a suspension batch cultures were noted. The volumetric titre of recombinant protein in the control-fed perfusion cultures was determined to be ~2.65- fold higher than that achieved in the batch encapsulated cultures. The rIgG1 had a higher degree of complexity at the end of the extended growth period in the control-fed perfusion culture in comparison to batch encapsulated cultures. The importance of monitoring cell viability and optimising the rate of perfusion was noted by the occurrence of a decrease in glycan complexity, associated with the accumulation of dead cells and glycosidase release.

Chapter 1: Thesis Introduction: High Density CHO Cell Cultures: Improved Productivity and Product Quality; A Literature Review

Abbreviations:

ADCC	antibody-dependant cell cytotoxicity
APA	alginate-poly-L-lysine-alginate
Asn	asparagine
BHK	baby hamster kidney
CHO	Chinese hamster ovary
CMP-NANA	Cytidine-5'-monophospho-N-acetylneuraminica
CO ₂	carbon dioxide
CPP	critical process parameter
CQA	critical quality attribute
Cys	Cysteine
DNA	deoxyribonucleic acid
DHFR	di-hydrofolate reductase
DO	dissolved oxygen
Dol-P	dolichol phosphate
Dol-P-Glc	dolichol phosphate glucose
Dol-P-Man	dolichol phosphate-mannose
DOT	dissolved oxygen tension
ER	endoplasmic reticulum
EPO	erythropoietin
FDA	Food and Drug Authority
FSH	follicle stimulating hormone
FPP	farnesyl pyrophosphate
GA	golgi apparatus
GalNAc	N-acetyl galactosamine
GDP-Man	Guanosine DiPhosphate mannose

GFAT	glutamine;fructose-6-P amidotransferase
GlcNAc-PP-Dol	N-acetylglucosamine-phosphate
GlcNAc-1-phosphotransferase	N-acetylglucosamine-1-phosphotransferase
Gluc I	α -1,2 glucosidase I
Gluc II	α -1,3 glucosidase II
GnT	N-acetylglucosaminyl transferases
GP2	Glycoprotein 2
GS	glutamine synthase
HEK	human embryo kidney
IgG1	Immunoglobulin G1
IFN- β	interferon- β
IR	Infrared spectroscopy
IPP	isopentenyl pyrophosphate
LH	LH series 210
Man I	mannosidase I
Man II	mannosidase II
NADH	nicotinamide adenine dinucleotide
NANA	N-acetyl-neuraminic acid
NBS	New Brunswick Scientific
NaOH	sodium hydroxide
NGNA	N-glycolyl-neuraminic acid
Poly-P-P	polyprenyl pyrophosphate
O ₂	oxygen
OST	oligosaccharyl transferase
OTR	oxygen transfer rate
OUR	oxygen uptake rate
SASA	solvent accessible surface
TNF- α	tumor necrosis factor alpha
t-PA	tissue plasminogen activator

TGN	trans golgi network
UDP-Gal	uridine di-phosphate galactose
UDP-GlcNAc	uridine di-phosphate-N-acetylglycosamine
UDP-GNAc	uridine di-phosphate N-acetylhexosamine
UTP	uridine tri-phosphate

1.1: Emergence of mammalian cells for recombinant protein production

Biopharmaceutical products have been described as clinical reagents, vaccines, and drugs produced using modern biotechnology for *in vivo* diagnostic, preventive and therapeutic uses (Zhu, 2012). The first biopharmaceutical for medical use, insulin (humulin), was produced and approved in 1982. Since then the biopharmaceutical industry has matured rapidly (Walsh, 2010). Recombinant deoxyribonucleic acid (DNA) and hybridoma technologies are extensively employed to engineer biological systems to produce various classes of proteins including: 1) recombinant forms of natural proteins (e.g. human growth hormones), 2) derivatives of natural proteins and living systems (e.g. viral like vaccines and IgG fusion proteins, 3) viral vectors and plasmid vectors which act as carriers for vaccination or gene therapy, 4) *in-vivo* diagnostic and therapeutic monoclonal antibodies. Since the approval of humulin, it has been reported that the Food and Drug Authority (FDA) have approved more than 100 new recombinant protein therapeutics and more than 300 non recombinant biopharmaceuticals (Zhu, 2012).

A wide array of biological systems for the production of the above pharmaceuticals exists including, microbial cells, insect cells and transgenic animals and plants. However mammalian cells exist as the host cells of choice for the commercial production of therapeutic proteins (Kim et al., 2012). According to FDA information, among the 27 latest approved biopharmaceuticals approved (January 08-June 11), 18 are recombinant proteins using cells, organisms or animals. Of the 18 recombinant proteins approved, 12 are produced in mammalian expression systems. It has been estimated that two third of the revenue generated in the biopharmaceutical industry comes from products manufactured using mammalian systems (Zhu et al., 2012).

Mammalian cells possess the cellular organelles and enzymes required for performing the post translational modification necessary for production of proteins in an active form. Pharmaceutical proteins do not consist of a polypeptide chain only. During protein

biosynthesis they usually undergo a series of co- and posttranslational modifications. Carbohydrate groups are covalently attached to the polypeptide backbone. Glycosylation, which involves the introduction of carbohydrate chains to proteins, is desired from a regulatory point of view (Nahrgang, 2002). A protein molecule is often defined in terms of its carbohydrate structures. Such post translational modifications were not previously possible when microbial cells were used as expression vectors. Microbial cells do not also display the capabilities to produce high molecular weight proteins but rather small proteins (Vives et al., 2003; Sakai et al., 2002).

Stably transfected mammalian cells were first generated by non-viral gene delivery in the early 1980's. The first therapeutic protein produced from a recombinant mammalian cell line to gain market approval was human tissue plasminogen activator (t-PA) (tPA, Activase; Genentech, S. San Francisco, CA, USA) (Kim et al., 2012). The expression system for this therapeutic was a Chinese hamster ovary (CHO) cell line derived from partial inbred female Chinese hamster (De Jesus and Wurum, 2011). Since then there has been a marked increase in the use of mammalian cells for the production of recombinant therapeutic proteins. Among the capable mammalian cells used, those documented as being of commercial importance include baby hamster kidney (BHK), mouse myeloma-derived NS0, human embryonic kidney (HEK)-293 and the human retina-derived PerC6 (Kim et al., 2012; Chon and Zarbis-Papastoitsis, 2011; Wurum, 2004; Barnes et al., 2003). It is however estimated that nearly 70 % of all recombinant therapeutic proteins produced today use CHO cell expression systems (Kim et al., 2012).

CHO cells are deemed the optimal mammalian cell line of choice for the production of new recombinant therapeutic proteins due to a number of desirable qualities: 1) CHO cell lines have been used as expression systems for the production of approved therapeutic recombinant proteins over the past 2 decades establishing them as safe hosts. Their safe host status makes them an attractive choice of expression system due to the associated ease of regulatory approval for new therapeutic proteins produced in these cell lines. 2) Mammalian cells, including CHO cells, have traditionally been associated with low specific productivity of recombinant proteins. Reliable and efficient gene amplification systems, including dihydrofolate reductase (DHFR)-mediated and glutamine synthase (GS)-mediated systems for CHO derived cell lines have overcome this drawback. 3) Mammalian cells in general are an attractive expression system due to their ability to perform post-translational modifications for production of proteins in a stable and active form. CHO cells in particular have the

capacity to efficiently produce recombinant proteins with glycoforms that are both compatible with and bioactive in humans. CHO cells possess genes for 99 % of the human glycosylating enzymes. It has been determined that 141 of these genes are not active during typical exponential growth (Butler and Meneses-Acosta 2012). However the glycoproteins produced by this cell line are deemed to be sufficiently similar to human forms. 4) CHO derived cell lines have been successfully adapted for growth in regulatory-friendly serum-free suspension cultures. Their ability to grow in suspension makes them the preferred candidate for large-scale culture in bioreactors (Kim et al, 2012; Butler and Meneses-Acosta 2012).

Since the emergence of the biopharmaceutical industry and CHO cells as expression hosts, a vast amount of research has gone into optimising growth and recombinant protein production of these cells in culture. With reported increases in the biopharmaceutical market of 35 % since 2001, the popularity of CHO cells as the host of choice for commercial production of therapeutic proteins is likely to continue (Kim et al., 2012). Optimisation of recombinant protein yields using a mammalian cell line, such as CHO, may be achieved through a variety of host cell engineering strategies (Hammond et al., 2012). Both the manipulation of single genes and clonal selection for desirable traits have proven to be successful applications in the progression towards product titre optimisation using CHO cell lines (Xu et al., 2011). Up until recently the genome sequence of CHO cell lines has been unavailable as a necessary tool for the application of genome-scale techniques for the generation of hyper productive cell lines. As of December 2011, the CHO genome database contains information of the ancestral CHO-K1 genome (Xu et al., 2011) and is accessible at <http://www.chogenome.org>. The availability of the CHO genome sequence represents another “tool in the bioprocessing toolbox” facilitating the 1) the design of targeted genetic manipulations 2) aid the revelation of components of poorly characterised phenotypes and 3) allow for a more thorough utilisation of ‘omic tools for CHO cell lines (Xu et al., 2011). The publication by Xu et al., 2011 has demonstrated how the availability of the CHO genome allows for insights into protein glycosylation capabilities and viral susceptibility of the CHO-K1 cell line. The CHO genome database has accommodated a movement of the CHO community (both academic and industrial) into the genome era facilitating the rapid output of information expected to emerge from efforts in the research community to sequence individual CHO cell lines (Hammond et al., 2012).

Alternative to or in combination with the manipulation of host cell genome for improved recombinant protein expression, manipulation and optimisation of upstream cell culture

conditions has also proven successful. Over the past 2 decades, a more than 100-fold yield improvement of titres in CHO cell culture has been largely attributed to the development of upstream cell culturing techniques (Jain and Kumar, 2008). Any parameter which affects the behaviour of mammalian cells at the upstream level may be termed a critical process parameter (CPP). The behaviour of cells covers all cellular activities including cell growth, metabolism, recombinant protein productivity and recombinant protein quality. The CPP's affecting the cell growth and recombinant protein yields at the upstream level include temperature, pH, dissolved gases (oxygen (O₂) and carbon dioxide (CO₂)), medium osmolality and composition, feeding strategy employed etc. All of such parameters have been extensively investigated and their implications on cell growth and productivity reviewed (Section 1.2).

1.2 Factors effecting high cell density growth and high productivity of cells

1.2.1 Temperature

Early studies investigating the effects of temperature on CHO cell growth and productivity were primarily concerned with optimising the maximum specific growth rate of the cells in culture. Kurano et al. (1990) conducted studies assessing the effects culture temperature may have on the specific growth rate of CHO cells. The optimum temperature for growth was documented as being 37 °C. Although the main focus of the study was to demonstrate an optimum temperature for cellular growth, such an initial study did give some indication that the cells did not display maximum specific productivity rates at this optimum growth temperature.

Subsequent studies therefore focussed on the effect culture temperature may have on a range of cellular characteristics such as the growth, viability and the specific productivity of the cells. Furukawa and Ohsuye (1997) conducted studies, involving the cultivation of a CHO cell line at temperatures of 30, 32, 33.5, 35, 36 and 37 °C. As previously demonstrated maximum cell growth occurred at high temperatures such as 36 and 37 °C. It was however demonstrated that maximum productivity occurred at 32 °C. Similar research however, carried out on different mammalian cell lines, did not indicate the same beneficial effects of low temperature cultivation regarding the productivity of cells in culture. Wiedemann et al. (1994) demonstrated that low culture temperature had no effect on the productivity of recombinant BHK cells producing antithrombin III.

This study and similar studies have led to the development of a “biphasic process” with “temperature shift optimisation” as demonstrated by Fox et al. (2003). During a biphasic process cells are typically cultured at 37 °C for a period of time. During this time high growth rates are demonstrated thus yielding high cell numbers capable of producing the recombinant protein. After high cell numbers have been achieved, the cultivation temperature is shifted to a lower temperature, 30-32 °C, thus inducing maximum specific productivity in the cells. Furukawa and Ohsuye (1999) demonstrated the beneficial effects of biphasic cultivation by shifting culture temperature from 37 °C to 32 °C during mid-exponential phase of growth. Recombinant protein production by the cells improved by 1.7-fold and 2-fold in comparison to the results obtained when the culture temperatures were remained at 32 °C and 37 °C, respectively, for the duration of the culture period.

The focus of subsequent studies, moving forward from research published by Furukawa et al. (1997 & 1999), has been to determine an optimum time frame for which to shift the temperature. Such studies have been completed by Fox et al. (2003), which focused on predicting a model for an optimum time to shift the culture temperature in order to maximise the production of the recombinant protein IFN- γ . It was demonstrated that shifting the culture temperature from 37 °C to 32 °C after 3 days, an increase in volumetric productivity of 40 % and 90 % was achieved compared to when the cells were cultured at 32 °C and 37 °C, respectively, for the entire cultivation period. It was noted that the vast majority of the recombinant protein was produced during the second half of the culture when the cell density was highest as expected.

Fox et al. (2003) have however demonstrated that this biphasic model for optimisation of productivity in CHO cells does seem to be cell line specific and that some cell lines, such as those demonstrated by Wiedemann et al. (1994) demonstrate no increase in productivity at lower culture temperatures, 30-32 °C. There has therefore been an interest portrayed in the adaption of CHO cell lines to hypothermic growth so as that the culture may be maintained at low cultivation temperatures for the entire cultivation process. Mammalian cells have showed an ability to improve their growth when subjected to changing environmental conditions. Yoon et al. (2006) established a study which would exploit this characteristic and alleviate growth suppression of CHO cells at low culture temperatures. The adaptation of two CHO cell lines to growth and productivity at 32° C was investigated. After adaptation of the two cell lines to low temperature growth the specific growth rates of the cell lines were increased

by 73% and 20% respectively. However the specific productivities were decreased by 49% and 22% respectively.

Sunley et al. (2005) have conducted studies investigating the effects of a combination of cell adaptation as well as biphasic cultivation on cell growth rates and productivity. Cells adapted to hypothermic growth were grown under low temperature conditions in batch mode for 10 days and demonstrated enhanced growth rates yielding a 6-fold increase in cell numbers compared to non-adapted cells grown under similar culture conditions. When adapted cells were subjected to biphasic cultivation a 4-fold increase in cell number was demonstrated by the adapted cells compared to the non-adapted cells. Adapted cultures produced a 2.6-fold increase and a 3-fold increase in specific productivities when grown in batch cultures and biphasic cultures respectively in comparison to non-adapted cultures.

A biphasic mode of operation allows for the generation of high cell numbers in the culture. Once high cell numbers are achieved it allows for the conditions to be shifted to provide an environment which allows the cells to exhibit maximum specific production rates resulting in a high concentration of recombinant protein product at the end of the culture period.

1.2.2 pH

Another important environmental factor which influences the behaviour of CHO cells in culture is the pH of the environment in which they are growing in. Early studies conducted by Kurano et al. (1990) investigated the effect of pH on cell proliferation and concluded that cellular growth may occur between a pH range of 7.0 to 7.9 with maximum growth occurring at pH 7.6. No focus however was given in this early study as to determining an optimum pH at which cellular productivity is at a maximum. It was determined by Trummer et al., (2006), that the optimum pH for growth of the cell line was 7.10. In terms of productivity, the specific production rate remained unchanged at different pH levels. However the maximum amount of product produced by the cell line occurred at pH 6.8 where the maximum concentration of the product produced was 1.8-fold higher than that at 7.1. This is due to the fact that increased cultivation time was observed at pH 6.8. Again it may be noted that the optimum pH for achieving high cell numbers is somewhat different to the optimum pH for product production. Similar results were noted from studies completed by Yoon et al. (2004). It was noted from such studies that a pH shift may also be incorporated into biphasic cultivations illustrating temperature shift optimisation. The implication of shifting the temperature to 33 °C and the pH to 6.9 after 3 days of culturing the cells at standard

conditions resulted in achieving the highest maximum viable cell concentration. These results were put forward in comparison to controls in which the temperature and pH remained at standard levels, as well as other cultures in which temperature was decreased and pH was increased.

It does appear that pH only effects CHO cell growth and productivity when the temperature of the culture is varied. It is therefore possible to suggest that optimum CHO cell growth occurs at pH levels varying around neutral and that culture temperature effects optimum growth and productivity of the cells to a greater extent (Borys et al., 1993).

1.2.3 Osmolality and concentration of dissolved carbon dioxide

Carbon dioxide may be described as a waste product produced by actively respiring cells in culture due to catabolic reactions. For every 1 mole of oxygen consumed, approximately 1 mole of carbon dioxide is released into the medium (Kimura & Miller, 1996). Carbon dioxide is also required to some extent by actively growing cells for the synthesis of pyrimidines, purines and fatty acids by the cells. As carbon dioxide is released by the cells it becomes dissolved in the culture media and causes a decrease in the pH of the culture media due to the following reaction.



The adverse effects of decreased pH on cell growth and productivity have been noted above. If however the pH is controlled by the addition of base, such as sodium hydroxide (NaOH) a subsequent increase in osmolality often occurs (deZengoitia et al., 1998). A physiological range between 31-54mm Hg of dissolved carbon dioxide is acceptable for maintaining the pH of the medium at an appropriate level and thus the necessity for increasing the osmolality of the medium due to NaOH addition is avoided (deZengoitia et al., 1998). Various studies have as such been carried out investigating the detrimental effects increased concentrations of dissolved carbon dioxide and increased osmolality may have on cultured CHO cells. It must be noted that cell culture medium is typically designed to have an osmolality between 260-320 mOsm/kg (Ozturk and Palsson, 1991).

deZengotita et al., (1998) have conducted studies which investigated the effects of various combinations of dissolved carbon dioxide levels and varying levels of osmolality on the growth rate of hybridoma cells producing monoclonal antibodies. The studies indicated that

higher dissolved carbon dioxide levels were associated with inhibition of cell growth and the onset of cell death. Such results were much more severe when there was no compensation made to the osmolality of the original media for increases in osmolality due to pH adjustments. Studies completed investigating the effects of osmolality alone with controlled dissolved carbon dioxide levels demonstrated a decrease in the growth rate of the cells by 86% in comparison to the growth rate observed for cells cultured in medium at an osmolality of 342 mOsm/kg. Kimura and Miller (1996) however demonstrated that increased osmolality had an insignificant effect on the growth rate of CHO cells in the presence of controlled dissolved carbon dioxide levels.

The study conducted by deZengotita et al. (1998) also indicated that the specific productivity of the cells was not in actual fact affected by elevated dissolved carbon dioxide or osmolality levels. One interesting observation made was the tendency towards higher specific productivity rates by the cells at elevated osmolality levels when the carbon dioxide level remained at 36 mm Hg. Kimura and Miller (1996) demonstrated that when the osmolality was permitted to change as the dissolved carbon dioxide level increased, the specific productivity remained the same or even increased for dissolved carbon dioxide levels of 195 and 250 mm Hg.

The general acceptance is that increased levels of osmolality do lead to increases in the specific productivity of the cells. However more recently Zhu et al. (2005) have documented that the increased rate of productivity of the cells at higher osmolality levels does not lead to an increase in the concentration of the product produced. This is due to the suppressed growth levels demonstrated by cells under conditions of high osmolality. The reduced specific growth rate of the cells at higher osmolalities has led to a reduction in the total number of viable cells producing the recombinant protein product. Subsequently when the specific productivity was seen to increase at higher osmolalities, no changes were evident in the concentration of product produced.

1.2.4 Concentration of dissolved oxygen

As with all other environmental cultural conditions, the concentration of dissolved oxygen (DO) in a cell culture medium has been demonstrated to be essential for optimising cell growth and productivity. Trummer et al. (2006) conducted studies investigating the effects of DO concentrations ranging from 10-100 % air saturation. It was noted that growth and viability were not broadly affected by the varying DO levels in the media and that the

specific rate of productivity of the cells also remained fairly constant within such a range. However it was noted that the viable cell density of the cells was reduced at lower DO concentrations such as 10 % due to the limited amount of oxygen available in the medium for growth and metabolism. Other studies have also demonstrated that when the DO concentration of a culture medium drops to zero, cellular growth ceases (Shi et al., 1993). The reduced viable cell concentration would result in the generation of a lower concentration of the recombinant protein due to the reduced numbers of cells producing the recombinant protein.

The % DO is determined by the efficiency at which oxygen may be transferred from the surrounding environment into the culture medium. All culture environments are aerated either through the contact of the surface of the medium with air, or through the sparging of the culture medium with bubbles containing air. The rate at which oxygen is transferred from the gaseous phase into the liquid medium is termed the oxygen transfer rate (OTR) and is denoted by the following equation:

$$N_A = k_L a (C_{AL}^* - C_{AL}) \quad (1.2.4.1)$$

N_A is the rate of oxygen transfer per unit volume of fluid, k_L is the liquid-phase mass transfer coefficient, a is the gas-liquid interfacial area per unit volume of fluid, C_{AL} is the concentration of oxygen in the broth and C_{AL}^* is the oxygen concentration in the broth in equilibrium with the gas phase. It is the difference between $(C_{AL}^* - C_{AL})$ which acts as the driving force in order for oxygen to dissolve in the media (Doran, 1995).

As noted from the equation above, the volumetric mass transfer coefficient is of great importance in determining the OTR. This coefficient may be influenced by some of the parameters which have previously been demonstrated in this review to affect the productivity of cells in a culture medium. Such parameters include the temperature of the medium, the osmolality of the medium, medium composition and cell numbers present. Analysis of the above equation does indicate that the gas-liquid interfacial area is an important factor in determining the volumetric mass transfer coefficient. It is desirable that the method of aeration used to increase the DO concentration in the culture medium maximises the interfacial area in order to maximise the OTR. As indicated previously, many culture mediums are aerated by spargers. Different types of spargers may be employed to produce different sized air bubbles which are as such broken up by the impeller and dispersed throughout the culture medium. The formation of small bubbles is desirable as they have

been noted to increase the interfacial area (Doran, 1995). Micron-sized bubbles have been demonstrated to induce a 10-20 fold increase in the OTR in comparison to larger bubbles up to 5mm in diameter (Zhang et al., 1992).

The OTR is also influenced by media components as they may affect the way in which bubbles behave in a culture medium. When small bubbles collide they may join together/coalesce to form larger bubbles, thus decreasing the interfacial area and the overall volumetric mass transfer coefficient. The inclusion of salt in culture media has been noted to prevent bubbles from coalescing (Doran, 1995). Oxygen transfer coefficients have been noted to be higher in 0.8 M salt solutions as oppose to just water alone (Zhang et al., 1992). The OTR however has been noted to decrease with increasing medium complexity. Antifoam may be incorporated into cell culture media in order to reduce the build up of foam in culture systems. Foam may be caused under agitation by the presence of proteins, polysaccharides and fatty acids in the culture medium. Antifoaming agents have been noted to reduce the surface tension of the bubbles, thus reducing the bubbles size and increasing the interfacial area. This principle however has not always lead to increases in the oxygen transfer rate as antifoam also reduces the mobility of the bubbles which subsequently reduces the mass transfer coefficient (Doran, 1995).

Temperature affects both the volumetric mass transfer coefficient and also the solubility of oxygen in the culture medium. Increasing the temperature has been noted to decrease the mass transfer driving force, $(C_{AL}^* - C_{AL})$, which in turn decreases the OTR. However increased temperature has been demonstrated to increase the diffusivity of oxygen in the liquid film surrounding the bubble and thus increasing k_L . There is therefore a variable effect of the two on the OTR depending on the temperature (Doran, 1995). The OTR in water has been demonstrated to remain constant over a varying temperature ranges due to the different effects different temperatures may have on decreasing the mass transfer driving force and increasing the volumetric mass transfer coefficient (Vogelaar et al., 2000). In regards to culture media, in general a temperature of 10-40 °C increases the OTR, whereas above 40°C the solubility of oxygen is affected decreasing the driving force for oxygen transfer. The presence of cells in the culture medium also influences the OTR by a process referred to as interfacial blanketing. Cells may adsorb at gas-liquid interfaces which reduces the area available for contact between the gas and the liquid (Doran, 1995).

In order for the cells to avoid a situation in which their growth may be affected by a limited supply of oxygen as demonstrated by Trummer et al. (2006), it is necessary to ensure that the

rate at which oxygen is utilised by the cells is equal to or less than the OTR. The rate at which oxygen is utilised by the cells is referred to as the oxygen uptake rate (OUR) (Doran, 1995). Although animal cells have been noted to have a lower specific OUR than that noted by microbial cells in culture, the oxygen demand of mammalian cells in culture has been illustrated to increase understandably when the cell concentration increases and also when the culture scale increases (Zhang et al., 1992). The OUR is therefore determined by the specific oxygen uptake rate of the cells as well as the number of cells present in the culture. This statement may be summarised by the following equation:

$$Q_o = q_o x \quad (1.2.4.2)$$

Culture states may be defined in terms of the level of dissolved oxygen, or % air saturation, which has been transferred into the culture medium. Due to the fact that the OTR must be greater than the OUR, a critical level of oxygen must be contained within the culture medium at all times, which is referred to as C_{crit} . If the oxygen concentration in the medium is below the level of C_{crit} , oxygen supply to the cells is limited and hypoxia/anoxia conditions exist. The desirable value for C_{crit} depends on the particular type of organism being cultured; however it usually lies at a level of 5-10 % air saturation (Doran, 1995). Normal or normoxic conditions exist when 100% air saturation exists. It has been demonstrated that no differences exist in the OUR demonstrated by the cells when the DO level varies from 10-150 % air saturation (Jan et al., 1997). However it has also been demonstrated that the OUR becomes supply-dependent below C_{crit} (Lin and Miller, 1992). The OUR may also be sensitive to the type of substrate present as an energy source in the medium. Glucose is consumed more rapidly than other sugars or carbon energy sources and thus the OUR is higher when glucose is used. Supplementation of a culture medium with glutamine or a mixture of amino acids resulted in no increase in the OUR being demonstrated by the cells. However when the culture medium was supplemented with glucose or the cells were re-suspended in fresh culture medium, the OUR increased by 40 % (Jan et al., 1997). As expected the OUR has also been demonstrated to increase when the cells are engaging in maximal productivity. Link et al. (2004) demonstrated that the maximum specific rate of productivity was achieved by the cells at 40 % DO. The OUR was at a maximum at this particular point in comparison to when the DO concentration was 5, 60 or 95 % and specific productivity was not at a maximum.

1.2.5 Concentration of essential nutrients and metabolic by-products

Animal cells require many essential nutrients for growth and survival in culture. Such nutrients include glucose, glutamine, essential amino acids, vitamins and serum components. The absence of one or more essential nutrient may result in cell death and therefore a decrease in the accumulation of the recombinant protein product (Xie and Wang, 2006). A basic medium for animal cell growth is Eagle's minimal essential medium. However due to the fact that over 30 essential nutrients are necessary for cell survival, many experiments have been carried out in order to fortify this basic medium to ensure that all nutrients are of a sufficient concentration so as that no one nutrient is depleted, which may result in cell death (Xie and Wang, 1997). Most of the literature investigating optimisation of nutrient components in animal cell culture media has focussed on the depletion of glucose and glutamine from the culture medium. Glucose is often present in cell culture media as the carbon energy source and glutamine is present as the nitrogen source (Xie and Wang, 2006; Kurano et al., 1990).

The concentration of glucose present in commercially available cell culture media ranges from 1-10 g/L (Sigma Aldrich, 2012). Of the commercially available cell culture media formulated for CHO cell growth in particular, the typical glucose concentration varies in the range of 3-5 g/L (Sigma Aldrich, 2013). When glucose concentrations are high, aerobic cells undergo glycolysis at higher rates than normal. Such an activity has been documented to result in the production of high levels of nicotinamide adenine dinucleotide (NADH) as well as the organic substrate pyruvate. The large amounts of NADH generated cannot be reoxidised and therefore the NADH may be utilised by the cells to drive the reduction of pyruvate to lactate in order to maintain homeostasis in the cell. The enzyme which catalyses this reaction is the lactate dehydrogenase enzyme (Matthews et al., 2000). The production of lactate from pyruvate may be summarised in Figure 1.2.5.1.

Typical concentrations of L-GLN in mammalian cell cultures vary in the range of 1-7 mM, depending on cell type and line (Freshney, 1987; Hayter et al., 1991). The utilisation of L-GLN involves both anabolic, located in the cytosol, and catabolic reactions (glutaminolysis), the latter of which occurs in the mitochondria (McKeehan, 1986). Anabolic metabolism is required for the generation of the building blocks for biomass and product formation, thus allowing L-GLN to serve as a protein constituent. Catabolism is mainly concerned with the generation of energy in the form of adenosine tri-phosphate (ATP) and is associated with the accumulation of ammonia in culture media (Vriezen et al., 1997).

The catabolism of L-GLN, summarised in Figure 1.2.5.1 below, initiates with the enzymatic removal of the amido group of L-GLN, yielding glutamate (L-GLU) and 1 mole of ammonia. This reaction is catalysed by the phosphate-dependent enzyme glutaminase. An alternative pathway for the amido group involves the purine and pyrimidine biosynthetic pathway in which it serves as an amino group donor for enzymes involved in such reactions. However, its catabolic fate lies in the production of α -ketoglutarate through cleavage of the α -amino group catalyzed by two possible enzymes, glutamate dehydrogenase and aminotransferase. The action of glutamate dehydrogenase yields a second mole of ammonia. The resulting α -ketoglutarate subsequently enters the citric acid cycle, where it is completely oxidised to carbon dioxide or partly oxidised to aspartate via transamination of oxaloacetate. Other possible outputs from the citric acid cycle include alanine and lactate, through the transformation of malate to pyruvate, brought about by the action of malate dehydrogenase. The action of the aminotransferase enzyme results in the yield of aspartate or alanine mainly through the transfer of the amino group to an α -keto acid, such as pyruvate or oxaloacetate (Schneider et al., 1996).

The theoretical yield of ammonia resulting from the catabolism of 1 mole of L-GLN is 2 moles. However due to the alternative possible pathways for both the amido group and amino group described above, the actual yield is ammonia may be lower (Vriezen et al., 1997). It must also be considered that the fate of L-GLN nitrogen is dependent upon the cell line and concentration of other metabolites in the culture such as glucose and other amino acids. Mammalian cell lines are derived from different species and organs and so therefore differ in specific enzymatic and metabolic patterns, as reviewed by Schneider et al., (1996).

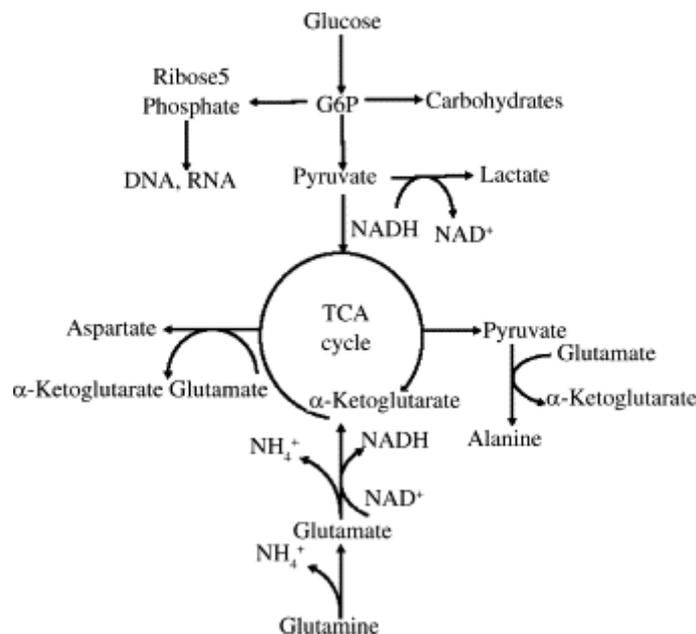


Figure 1.2.5.1: Overview of the metabolic fates of glucose and glutamine in mammalian cells, from Teixeira et al., (2005).

The irreversible chemical degradation of L-GLN to form pyrrolidonecarboxylic acid in aqueous solutions, such as cell culture media, also results in the build up of ammonia in cultures. The reaction follows first order kinetics and is characterised as an intramolecular SN₂ reaction in which the free electron pair of the α-amino nitrogen attacks the carbonyl carbon of the final amido group. After the formation of a tetraedric transition state, the amido nitrogen is released as ammonia with the formation of pyrrolidonecarboxylic acid. For every one mole of L-GLN degraded, one mole of ammonia is released. The reaction is dependent upon a number of factors including solution temperature, pH and other components in the solution (Schneider et al., 1996).

In an effort to reduce the accumulation of ammonia in the culture media, a number of strategies have been employed. Schneider et al., (1995) reviewed methods to physically remove accumulated ammonia such as (1) the use of gas-permeable, hydrophobic porous membranes; (2) application of non-porous ion-exchange membranes; (3) use of ion-exchange resins; (4) electro dialysis. However the application of such methods is limited in that the materials are selective for ammonia removal only. More biological compatible approaches involve reducing the concentration of ammonia accumulated in the culture environment. This may be brought about by designing culture feed-strategies which allow for lower initial L-GLN concentrations thus avoiding chemical breakdown of excess L-GLN present in cultures (Wong et al., 2005, Wang et al., 2002). More recently efforts have been made to establish

CHO cell lines which are able to grow in completely L-GLN free medium (Taschwer et al., 2012).

Various studies have documented the negative effects of ammonia and lactate on cell growth and productivity. These effects include an increase (ammonia) or a decrease (lactate) in the pH of the medium (Miller et al., 1987). Ammonia has been illustrated to permeate the cell wall and partition into cellular compartments, disrupting the internal pH which also has adverse effects on cellular growth and productivity (Bibila and Robinson, 1995). The accumulation of ammonia limits the growth and productivity enhancement possibilities of high L-GLN concentrations. Canning and Fields, (1983); Hansen and Emborg, (1994); Yang and Bulter, (2000) have all reported a negative effect of an ammonia concentration as low as 2 mM on cell growth and recombinant protein production. High lactate concentrations, induced by the addition of 60 mM sodium lactate have been noted to inhibit growth in CHO cells by 25 % (Lao and Toth, 1997). At the end of the cultivation period in hybridoma cultures, the concentration of lactate was noted to be 63 mM which was thought to be associated with the onset of cell death (Zhang et al., 2004).

1.2.6: Feeding strategy

Four major methods of cultivation of animal cells have been documented, depending on the methods of providing culture medium containing nutrients to the cells: batch, fed-batch, continuous and perfusion (Xie and Wang, 2006). Batch cultivation of mammalian cells is considered one of the simplest modes of cultivation and is particularly effective when protein expression is growth associated (Gugerli, 2003). The persistent use of such a mode of cultivation is facilitated by the continuous development of high producing cell lines, developments in media formulations satisfying the metabolic demands of cells in culture, and also the ease of scalability (Bonham-Carter and Shevitz, 2011). Batch operations offer high flexibility, lower risk of contamination and more efficient process development and validation (Gugerli, 2003). One trait of mammalian cells in culture which is not satisfied by a batch mode of cultivation is the highly deregulated metabolism demonstrated by mammalian cells exposed to common culture conditions. When cells are extracted from animal tissue, where they are found in a highly controlled environment, and exposed to common culture conditions in bioreactors, they characteristically display consumption rates of the main carbon and energy sources, far above rates strictly required to give support to cell metabolism (Sanfeliu et al., 1997). The highly deregulated metabolism of the cells under such conditions,

results in rapid exhaustion of the culture medium, accumulation of toxic metabolic by-products (namely lactate and ammonia), resulting in the consequent initiation of programmed cell death or apoptosis very shortly after the attainment of maximum cell number. As a result of low cell numbers achieved and the rapid onset of cell death, low product titres and poor volumetric productivity are normally achieved (Leelavatcharamas et al., 1999). Fed-batch processes have been described as the most common modes of cultivation utilised for recombinant protein production in mammalian cell cultures and have been operated up to 20,000 L working volume, as reviewed by Chon and Zarbis-Papastoitsis, (2011). The fed-batch strategy seeks to avoid early complete depletion of key media components, common to batch cultivation, by feeding highly concentrated nutrient feeds to the culture at set points. Two methods for the optimisation of the concentration of nutrients in the feed medium have been proposed and reviewed. The first method is described as an iterative method in which cultivation begins in a near optimal basal medium. Concentrated nutrient solutions are subsequently added to the culture according to the requirements of the growing cells. Such requirements are determined by analysing the spent medium and determining cellular kinetics of growth and metabolite consumption and production. The second method is characterised by the addition of concentrated nutrient feed of complete medium and/or standard amino acid and glucose and glutamine as supplementation feed (Jain and Kumar, 2008). Once the culture reaches a pre-determined criteria, (e.g. culture time, percent viability, nutrient or metabolite concentration etc), the culture is terminated and the broth is processed to purify the desired recombinant protein (Chon and Zarbis-Papastoitsis, 2011). One of the major advantages associated with fed-batch cultivation is the relatively high concentration of final product present in the media at the point of culture termination (Hu et al., 2011). Therefore the yield of product in the medium is higher than that which would be observed in continuous or perfusion cultures. Fed-batch processes have been optimised to maximise parameters such as viable cell density, culture time, specific productivity, which determine the final concentration of protein in the media (Bibila and Robinson, 1995). However, as with any mode of cultivation, there are disadvantages associated with this method. The high cost of concentrated media, the expertise required and intensive laborious nature of the process are notably some (Jain and Kumar, 2008). Results are not assured and even after intensive optimisation of the process, only little benefit has been seen. Feeding strategies involving the addition of nutrients (such as glucose and glutamine) in highly concentrated formulations incorporates the risk of excessive medium osmolality which may inhibit culture productivity.

The addition of high concentrations of glucose may induce a Crabtree effect resulting in a shift to fermentative metabolism of the cells, resulting in increased lactate yields, limiting cell growth and productivity due to premature stress on cells (Jain and Kumar, 2008). However one of the major causes for concern is the residence time of the recombinant protein in the culture environment increasing the risk of the protein being exposed to proteases and glycosidase enzymes (Gramer and Goochee, 1993).

The problems associated with fed-batch modes of cultivation may be alleviated with the progression to a continuous or perfusion feeding strategy. The main advantages of continuous/perfusion modes of cultivation are that they can avoid nutrient limitation, while reducing the concentration of growth inhibitory metabolites (Yang et al., 2000 and Konstantinov et al., 1996). Product quality and purity may be enhanced, not only due to the reduced by-product concentration, but also as an effect of the continuous withdrawal of protein containing spent media from the culture environment, thus reducing the overall residence time of the protein in the culture environment in which it may be exposed to proteases and glycosidase enzymes. (Rastilho et al., 2002; Ryll et al., 2000).

In a continuous culture fresh medium is continuously fed while the spent medium is removed, thus maintaining the cells in a relatively optimal biochemical environment (Chon and Zarbis-Papastoitsis, 2011). The requirements of a continuous process have been outlined and include (1) the ability to reach a high cell density, (2) control of cell proliferation and (3) the prevention of apoptosis during the maintenance phase (Gugerli, 2003).

Continuous cultures are regulated by two main parameters (1) the feed rate or perfusion rate and (2) the bleed rate, which has been described as the flow rate of the cell-containing stream outgoing the reactor. A continuous culture is governed by the following equations:

$$P = \frac{F_P + F_B}{V} \quad D_B = \frac{F_B}{V} \quad \mu = D_B \cdot \frac{N_t}{N_v} \quad k_d = \mu - D_B$$

where P is the perfusion rate, F_P the cell free outgoing stream, F_B the cell-containing outgoing stream, V the reactor volume, D_B the bleed rate, μ , the growth rate, N_t the total cell number, N_v the viable cell number and k_d the death rate (Gugerli, 2003).

Cell concentration typically increases constantly until it becomes limited by the high cell population density. The high cell density attained leads to high volumetric productivity. If high cell density is accompanied by low viability, the overall result in an accumulation of dead cells. Therefore the key process parameters specific to the continuous culture are the feed and bleed rate, as well as feed composition (Jain and Kumar, 2008).

If $P = D_B$ the system is referred to as a conventional chemostat culture and is characterised by a growth rate which is equal to the dilution rate. The maximum cell concentration is subsequently governed by the following equation (Gugerli, 2003):

$$X_{\max} = (C_{i,F} - C_{i,out}) \cdot Y_{X/i} \cdot \frac{P}{D_B \cdot V}$$

Continuous chemostat cultures have been documented to sustain steady state operations over extended periods of time (Birch et al., 1987; Frame and Hu, 1991). However one of the shortcomings of this proposed method is the relatively low cell concentrations attainable at steady-state due to the necessary bleed rate (Vits and Hu, 1992). It is true that a bleed stream is necessary for not only removal of dead cells, but also to enable control of the culture and prevent overgrowth. However, continuous processes can only compete economically with batch cultures if a higher cell density and high protein concentrations can be reached. Cell concentration and volumetric productivity can be effectively increased by cell recycle or partial or complete cell retention. Coupling a cell retention device to a continuous bioreactor, in what is frequently called a perfusion bioreactor, can lead to a 5-10 fold increase in cell concentration (Vits and Hu 1992). A diverse set of devices have been developed over the years which enable the retention of cells in bioreactors, while minimising damage to the cells. Devices may be based on the principle of filtration, sedimentation and/or centrifugation. Some of such devices work better than other and have been extensively reviewed by Gugerli, (2003), Chon and Zarbis-Papastoitsis, (2011), Bonham-Carter and Shevitx, (2011) and Voisard et al., (2003).

In theory if cells are completely retained, no steady state should be reached and the cell concentration should go towards infinity. In practice, however, Gugerli, (2003) described how cells grow until one or several nutrients become limiting. This occurs when the nutrient feed rate is no more able to cope with the consumption rate required by the cells for either

growth or maintenance, resulting in the onset of apoptosis. A steady-state viable-cell concentration will be reached where the death rate equals the growth rate resulting in an accumulation of dead cells (Jain and Kumar, 2008). In the case where a constant perfusion rate is used and the bleed rate is 0, Gugerli, (2003) described the characteristic behaviours of cells, summarised as 4 distinct phases: (1) a first exponential growth phase due to excess nutrients (2) a second linear growth phase limited by the feed rate, (3) a decrease in growth due to insufficient nutrient feeding (4) slow cell death due to apoptosis. Therefore, once a suitable retention device has been proposed, the next issue is maintaining the high cell concentration over an extended period of time. In general it is understood that perfusion cultures usually approach steady state more rapidly than chemostat cultures without cell retention (Miller et al., 1988; Hiller et al., 1993) mainly because of much higher perfusion dilution rates due to greater cell concentrations.

The main criteria for consideration in a perfusion culture with retention are therefore: 1) the time point at which the perfusion is initiated 2) The magnitude of the perfusion rate applied 3) the extent to which the cells should be retained. Up until 2 decades ago, little information for the selection of such criteria was available (Vits and Hu 1992). Dowd et al., (2001) investigated the importance of perfusion initiation time on the success of a perfusion culture. It was found that when starting the perfusion at a time point when glucose was not limiting (8.5 mM), there was a 3-fold decline in cell specific productivity. It was assumed that even though glucose was not yet limiting, the culture was approaching a batch like stationary phase. When perfusion was initiated at an earlier time point (glucose concentration was ~ 14 mM), the same drop off in the cell specific production rate was not observed. In regards to the rate of perfusion, it is now understood that the main method for operating a perfusion culture is to adjust the perfusion rate in order to maintain the concentration of both key nutrients and by-products at a level suitable for the particular cell line used (Wang et al., 2002; Konstantinov et al., 1996; Goldman et al., 1998).

Meuwly et al., (2004) have proposed 3 approaches for consideration, which seek to achieve optimal medium perfusion rate: 1) operation of the culture with a fixed perfusion rate during the entire culture production run. This enables the system to run in a robust and defined manner in regards to medium costs. 2) perfusion rate is adjusted in response to either cell number or key nutrient (glucose and glutamine) consumption. This method is difficult to operate for cases where cell bleeding is not possible (such as immobilisation) and therefore may possibly result in an overgrown, out of control culture. 3) the final method consists of a

combination of 1) and 2) in which the culture is divided into two phases. The first phase, termed the growth phase, in which the perfusion rate is adjusted with cell growth, is followed by a shift in some other parameter to slow cell growth and reduce metabolism. According to a review by Bonham-Carter and Shevitz, (2011), it is due to these advances in manufacturing and perfusion culture operation that perfusion is the preferred method of operation for recombinant protein production, followed closely by fed-batch processes due to the advantage that have been listed above.

1.2.7: Immobilisation of mammalian cells for enhanced cells densities and higher productivities

The immobilisation of mammalian cells has found widespread application in many biotechnological fields. Microencapsulation was first used in the treatment of diabetes, in which pancreatic islet cells were first immobilised in alginate and delivered in vivo without the occurrence of a rejection reaction (Lin and Sun, 1980). Since then mammalian cell immobilisation has been used for various applications including the encapsulation of recombinant cells containing genes encoding therapeutic proteins. Injection in vivo of the cells contained in the capsules results in delivery of such gene products to the host. Genetically engineering cells delivering biologically active human factor IX (Liu et al., 1993), human growth hormone (Chang et al., 1993) and human adenosine deaminase (Hughes et al., 1994) have all been encapsulated successfully in alginate microcapsules to deliver the recombinant gene product (Awrey, et al., 1996). It is a promising strategy for the controlled localised and long term in vivo delivery of therapeutic proteins to the host (Ma et al., 2006).

Microencapsulation of mammalian cells has found widespread application in large scale cell cultures for the production of therapeutic proteins in vitro. Animal cells have a natural tendency to aggregate and so the immobilisation of cells in microcapsules to provide optimal shear-stress free conditions is considered a promising technique (King et al., 1987). Based on the first encapsulation of pancreatic islet cells in alginate beads, Lim and Moss, (1981) improved this method for the production monoclonal antibodies on a large scale. These initial reports suggested a 10-fold increase in monoclonal antibody (mAb) yield in immobilised hybridoma cultures in comparison to suspension cultures due to an increase in cell densities achieved in cultures, as reviewed by Selimoglu and Elibol, (2010). Mammalian cells are only protected by a membrane and in comparison to yeast and bacteria are fragile. As a result high

cell densities in suspension are limited by the mechanical and shear forces resulting from agitation and aeration in large scale bioreactors (Papoutsakis, 1991). Encapsulation creates a protective microenvironment for these fragile cells and is associated with a number of advantages over the cultivation of cells as free suspension cells, including (1) protection from damaging stresses induced by mechanical agitation and/or air sparging systems, (2) provision of a retention system for the application of perfusion strategies. Microcapsules/beads entrapping the cells are 50-1000 times larger than free-suspended cells and so do not clog conventional filter systems. (3) Higher local cell concentration and overall cell yield in the culture, in the order of 10^7 cells/ml, can be achieved, resulting in high volumetric productivities and reduced capital costs (Lee et al., 1993). Focusing on the concentration of cells within the microenvironment, a number of advantages have been associated with high localised cell densities leading to increased recombinant protein production. Information originating from a cell can easily be communicated to other cells which are in close proximity. The concentration of autocrine factors would be locally higher inside the immobilisation matrix, as opposed to being diluted in the surrounding media when cells are present in suspension. (Yamaguchi et al., 1997). Farrell et al., (1994) recognized that endogenous proteins derived from hybridoma cells contributes to increased mAb productivities. In particular the cytokine interleukin-6 (IL-6) stimulates mAb production in a number of hybridoma cell lines (Makishima et al., 1990). The immobilisation of cells in a matrix induces a physical limitation of space available for cell proliferation. Therefore reduced specific growth rates observed in immobilised cultures have been associated with higher specific rates of recombinant protein production. Yamaguchi et al., (1997) reported increased mAb production from baby hamster kidney (BHK) cells immobilised in collagen gel particles in the late stage of the culture due to a slower cellular growth rate. However mAb production was also enhanced at early culture stages when cellular growth rates remained high, in comparison to suspension cultures. Increased mAb production was associated with increased communication of cells locally. This hypothesis was verified by a separate study in which the concentration of production stimulating autocrine factors was increased in the media, and no significant increase in mAb production was observed. Mammalian cell immobilisation has achieved a combination of higher cell densities and higher volumetric productivities than those which would be achieved for suspension cultures for various authors ((Sinacore et al., 1989; Lee and Palsson 1990; Ma et al., 2006; Breguet et al., 2007; Selimoglu et al., 2012).

Immobilisation of mammalian cells in culture may be in the form of immobilisation on macroporous microcarriers and hollow fibre membranes or encapsulated within a gel matrix. Enhanced growth and viability have been associated with the application of macroporous microcarriers (Griffiths, 1990), but have been demonstrated to present diffusion limitations (Ng et al., 1995). The encapsulation of cells within porous microcapsules provides for the microenvironment needs of the cells (Breguet et al., 2007) and eliminates diffusion limitations associated with large capsules (>1 mm) (Glacken et al., 1983; Robitaille et al., 1999).

Various polymers such as chitosan, alginate and polyamino acids have been used in the formation of microcapsules (Zhang et al., 2007). An ideal immobilisation material should be (i) biocompatible and non-toxic (ii) allow for the sufficient diffusion of oxygen, metabolites and their waste products, secreted proteins (if secretion is desired), while preventing cells from leaking (iii) must be chemically and mechanically stable in the somewhat harsh large scale culture environment for long term processing, as reviewed by Selimoglu and Elibol, (2010). Alginate-poly-L-lysine-alginate (APA) microcapsules are reportedly the most widely studied microcapsules applied to mammalian cell cultures for enhanced recombinant protein production (Zhang et al., 2007). Alginate is a polysaccharide extracted from seaweed and is composed of homopolymeric regions of 1,4 linked β -D-mannuronic acid (M-blocks) and α -L-guluronic acid (G-blocks) residues. The M- and G-blocks vary widely in composition and sequence, depending on the organism and tissue they are located from. (Selimoglu and Elibol, 2010; Peirone et al., 1998). Alginate serves as the polymer of choice in many microencapsulation applications as it has already been licensed by the US Food and drugs authority (FDA) for application as a pharmaceutical excipient, a viscosity modifier in food and as a wound dressing (Bohari et al., 2011). It is formed under mild conditions and so is biocompatible and biodegradable (Selimoglu and Elibol, 2010).

The application of alginate in the formation of 3-D microcapsules containing actively growing mammalian cells involves the gelation of the polymer due to interchain chelation of divalent cations such as Ca^{2+} and Ba^{2+} . These cations competitively bind to preferentially G-blocks by an exchange of sodium ions from the G-blocks with the divalent cations. (Selimoglu and Elibol, 2010; Peirone et al., 1998). Each alginate chain can dimerize to form junctions with many other chains and as a result a gel network is formed, as oppose to insoluble precipitates. Ca^{2+} is the most suitable ion in relation to biocompatibility of mammalian cells to the ion and also affinity for the sodium alginate. The gelled bead size and

strength are related to both the molecular weight and concentration of the alginate used. Molecular weight determines the intrinsic viscosity of the sodium alginate. Using low viscosity alginates requires a high concentration for the formation of a convenient bead structure. Stabler et al., (2001) suggested a sodium alginate concentration between 1 and 1.5 % in order to provide sufficient mechanical stability with optimum mass transfer between the extra capsular environment and the cells found inside the gel. Capsules are predominately made from a concentration of 1.5 % w/v sodium alginate crosslinked with 1.1% (0.1 M) Ca^{2+} . The immobilisation of mammalian cells in alginate beads is brought about by the extrusion of a homogeneous sodium alginate solution containing a certain density of mammalian cells through a nozzle of known diameter. The application of vibrational technology to the nozzle results in the formation of universally sized spherical droplets, which become immersed in a batch of Ca^{2+} solution. The result is the formation of a gelled bead containing evenly distributed mammalian cells immobilised within the matrix. The beads are coated by a layer of poly-L-lysine (PLL) followed by a second layer of alginate, usually of lower concentration (0.03 %). PLL is a polycation, homopolymer. The alginate bead becomes coated with PLL due to the electrostatic attraction forces between the PLL and sodium alginate (Leick et al., 2011). PLL, with a range of molecular weights may be used, and subsequently determines the molecular weight cut-off of the microcapsule membrane. The purpose of the additional coating with 0.03 % alginate is to neutralise any un-reacted PLL on the surface of the gelled bead and improve the biocompatibility of the beads (Wang et al., 2005). The resulting solid bead structure containing the immobilised cells may be liquefied with sodium citrate or semi-liquefied, brought about by further incubation in Ca^{2+} . Liquefaction occurs through incubation in sodium citrate due to the subsequent chelation of the Ca^{2+} ions in the gel core (Selimoglu and Elibol, 2010). The results of such liquefaction are that cells float freely around the interior of the capsule (Gugerli, 2003). Alginate has been shown to support the growth of a range of different cell types including pancreatic islets, fibroblasts and myoblasts and is associated without a drastic loss of viability after immobilisation. It supports high cell seeding densities and remains stable and intact throughout long cultivation processes, therefore making APA microcapsules suitable for the expansion of high cell numbers in bioreactors in vitro (Bohari et al., 2011).

1.3: Quality of produced recombinant proteins

Over the past two decades a vast amount of progress has been made in the identification of culture conditions affecting cell growth and productivity. State of the art processes yield up to 6 g/L IgG, which represents a significant improvement from 1 g/L observed less than a decade ago. However, product yield should not be the sole parameter used for the optimisation of upstream processes. Attention must be paid to the fact that increased production levels may compromise the posttranslational modification machinery of the cell, yielding high levels of product with reduced activity, lower stability, and/or decreased batch-to-batch consistency (Van Berkel et al., 2009). The subsequent lack of rapid commercialisation of protein drug candidates in the biopharmaceutical may be attributed to the necessity to monitor and control a vast range of CPP's which have been demonstrated to affect not only recombinant protein production but also the quality (Wang, 2005). The main critical quality attributes (CQA) determining the biological activity of a recombinant therapeutic protein are the glycosylation and aggregation state of the protein.

As stated above, all proteins consist of a polypeptide chain. During protein biosynthesis these polypeptide chains usually undergo a series of co- and post-translational modifications. One such modification that plays an important role in the efficacy of therapeutic proteins is glycosylation (Jones et al., 2005) Glycosylation may be defined as an enzymatic process by which sugars (known as glycans) are chemically attached to proteins to form what are known as glycoproteins. While the proteome is coded in the genome, unlike the synthesis of proteins, these post-translational modifications are not directed by a template (An et al., 2009). They are very much dependent upon the influence of environmental factors on the activity of glycosylation enzymes (glycosyltransferases) and the availability of glycosylation precursors. As a result, glycoproteins are produced as pools of different glycoforms with varying glycan structures attached to the polypeptide backbone. Glycoproteins are therefore normally heterogeneous in nature. Macroheterogeneity refers to variation in the site occupancy of the attached glycans. Microheterogeneity refers to variations in the structure of the attached glycans (Jones et al., 2005; Butler, 2005).

A vast amount of research has gone into glycomics; controlling the pool of glycan structures that occupy a recombinant protein (Butler, 2006). The production of a recombinant protein as a biopharmaceutical product requires that a consistent glycosylation profile is maintained between batches. Therefore an understanding of the culture parameters affecting the glycosylation profile of a specific recombinant protein is essential. However before

understanding how to control the glycosylation profile of recombinant proteins, it is first essential to understand the biosynthesis of these nucleotide sugars and how they are attached to protein polypeptide chains (Butler, 2005).

1.3.1: N-glycan biosynthesis:

There are three known ways by which a glycan structure may be covalently attached to a polypeptide backbone: 1) via the NH₂ group of an asparagine residue, 2) via the OH-groups of usually serine or threonine, 3) or through an ethanolamine phosphodiester (Jenkins et al., 1996). The most common form of recombinant protein glycosylation is N-glycosylation and for that reason N-glycans are the most widely studied structural form of glycosylation (Hossler et al., 2009).

N-Glycans formation

All N-glycans produced by mammalian cells typically consist of a core pentasaccharide (Man₃GlcNAc₂) attached via an N-glycosidic bond to an Asparagine (Asn) residue of a consensus amino acid sequence. To be recognised, the Asn residue must be part of a tripeptide sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. The tripeptide sequence is the consensus amino acid sequence and is often referred to as a sequon. The position of the sequon in the polypeptide chain exerts an influence on the glycosylation of the protein. Sequons positioned in close proximity to the amino or carboxyl-termini have a tendency to be less efficiently glycosylated (Opdenakker et al., 1993).

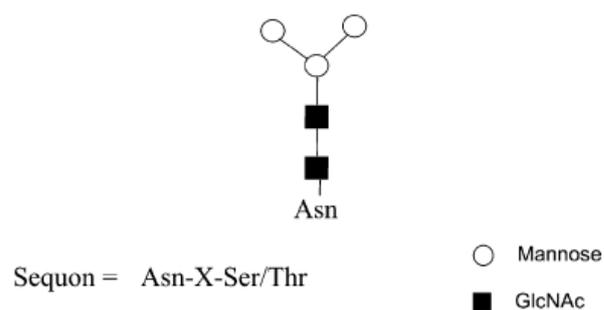


Figure: 1.3.1.1: Core N-glycan structure (Butler, 2006)

N-glycan biosynthesis and the occupation of a sequon occur co-translationally in the endoplasmic reticulum (ER). The process is initiated with the enzymatic transfer of a pre-synthesized 14-mer oligosaccharide structure (Glc₃Man₉GlcNAc₂) from a dolichol-linked

pyrophosphate donor to an Asn residue on the consensus polypeptide backbone of the protein.

Dolichol is formed as an end product of the prenyl biosynthesis pathway. It is described as a polyisoprenoid lipid with 80-100 carbons or 16-20 isoprene units and includes a saturated α -isoprene subunit. Dolichol synthesis is catalysed by *cis*-prenyl transferase. The enzyme is involved in the elongation of the soluble unsaturated pyrophosphate, farnesyl pyrophosphate (FPP) to the correct length using isopentenyl pyrophosphate (IPP). The result is the formation of a hydrophobic fully unsaturated polyprenyl pyrophosphate (Poly-P-P), which is embedded in the ER membrane. Dephosphorylation and reduction of the α -isoprene unit of the Poly-P-P culminates with the formation of the lipid glycosyl carrier, dolichol phosphate (Dol-P) (Jones et al., 2005). On the cytosolic side of the ER, the 14-mer oligosaccharide structure is synthesised through the step-wise addition of nucleotide sugars (e.g. uridine di-phosphate-N-acetylglycosamine (UDP-GlcNAc) and Guanosine DiPhosphate mannose (GDP-Man)) or lipid intermediates (e.g. dolichol phosphate-mannose (Dol-P-Man) and dolichol phosphate glucose (Dol-P-Glc)) to Dol-P to form N-acetylglucosamine-phosphate (GlcNAc-PP-Dol) (Butler, 2006). This reaction is catalysed by a membrane-associated glycosyl transferase, N-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase). Another GlcNAc and five mannose units are then transferred to GlcNAc-PP-Dol to form the intermediate $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$. This intermediate is then transferred to the luminal side of the ER where it is further extended by the sequential addition of four more mannose residues and three glucose residues (Jones et al., 2005).

The enzyme involved in the recognition of the Asn residue of a sequon and the subsequent transfer of the oligosaccharide is referred to as oligosaccharyl transferase (OST). This enzyme exists as a complex of several subunits which are bound to the ER membrane approximately 65 amino acid residues from the ribosome P-site. The entire N-glycosylation processing method can be seen in the figure below, right from the occupation of a sequon to complex glycan processing in the Golgi apparatus (GA) giving rise to glycan heterogeneity (Butler, 2006).

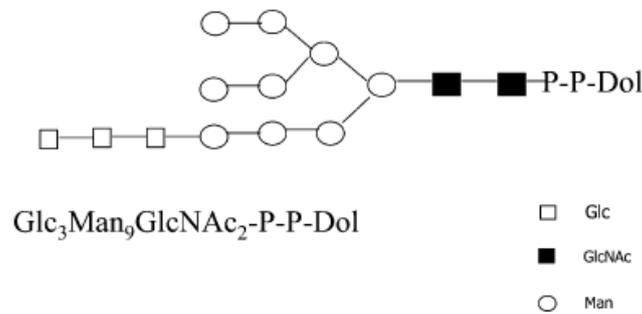


Figure: 1.3.1.2: N-glycan precursor attached to dolichol carrier (Butler, 2006).

1.3.1.1: N-glycan trimming in the ER

Glucosidases and Mannosidases located in the ER membrane engage in a process known as trimming of this native N-glycan structure. The trimming process is initiated by the enzyme α -1,2 glucosidase I (Gluc I) which is responsible for the removal of the terminal glucose unit on the 14-mer oligosaccharide structure. (S1) The next two glucose units are sequentially removed in a reaction catalysed by the enzyme -1,3 glucosidase II (Gluc II). Finally the glycan structure is trimmed to a Man₈GlcNAc₂ structure through the action of mannosidase I (Man I) before being translocated to the cis compartment of the GA (Opdenakker et al., 1993; Butler, 2006).

1.3.1.2: N-glycan processing in the golgi

In the GA, Man I activity continues, yielding a high mannose, Man₅GlcNAc₂, structure. This trimmed glycan structure under goes complex processing in the GA as a result of the variable activity of a series of enzymes. A series of transferase enzymes are responsible for the sequential addition of monosaccharide's including GlcNAc, galactose, fucose and sialic acid resulting in elongation of the N-glycan structure. The first of these enzymes are the N-acetylglucosaminyltransferases (GnT). They are responsible for the conversion of the high mannose structure into a complex structure through the introduction of variable antennarity onto the core structure. Their actions also allow for bisecting GlcNAc to occur between two antennae. As seen in Figure 1.3.1.2.1, GnTI transfers GlcNAc residues from the nucleotide sugar donor UDP-GlcNAc to the α 1,3 mannose arm of the high mannose structure. The resulting hybrid structure is converted to a Man₃ hybrid structure by the action of a mannosidase II (ManII) enzyme. GnTII then transfers a GlcNAc residue to the free α 1,6 mannose arm of the hybrid structure. The resulting structure is a complex biantennary glycan. Before elongation of the complex glycan through the action of galactosyl transferase and sialy transferase enzymes, other GnT's may introduce further branching in the glycan, as

illustrated in figure below. The combined activity of GnTIV and GnTV leads to a tetra-antennary structure denoted by M₃Gn₄, Figure 1.3.1.2.2 (Butler, 2006).

The action of the GnT enzymes is followed by fucosylation of the complex biantennary glycan. This reaction is catalysed by the enzyme α 1,6 fucosyltransferase, which is a type II transmembrane protein. It is responsible for the transfer of fucose from the fucose donor GDP-fucose to the Asn-linked GlcNAc residue. Following fucosylation, the N-Glycans are transported to the trans compartment of the GA where the emerging glycan structure is subjected to the action of galactosyl and sialy transferase enzymes (Butler, 2006).

Sialylation is the last intracellular stage of the glycosylation process. The sialylation process involves the transfer of sialic acid from the nucleotide sugar precursor, Cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-NANA), to galactose residue on the glycan structure. This reaction is catalysed by the sialy transferase enzyme. 18 different types of sialy transferase enzymes have been cloned from animals thus giving rise to various terminal linkages between the sialic acid and the galactose residues. The two most important linkages for human glycoproteins are the α 2,3 and the α 2,6 linkages. These two reactions are catalysed by α 2,3 and α 2,6 sialy transferases respectively. The α 2,6 sialy transferase enzyme is absent in hamsters and so CHO cells produce glycoproteins with α 2,3 terminal sialic acid residues only (Butler, 2005; Jenkins et al., 1996).

Following sialylation in the trans part of the GA, the glycoproteins are transported to the trans golgi network (TGN) from which they emerge in vesicles to transport the assembled glycoprotein to its target (Nahrgang, 2002).

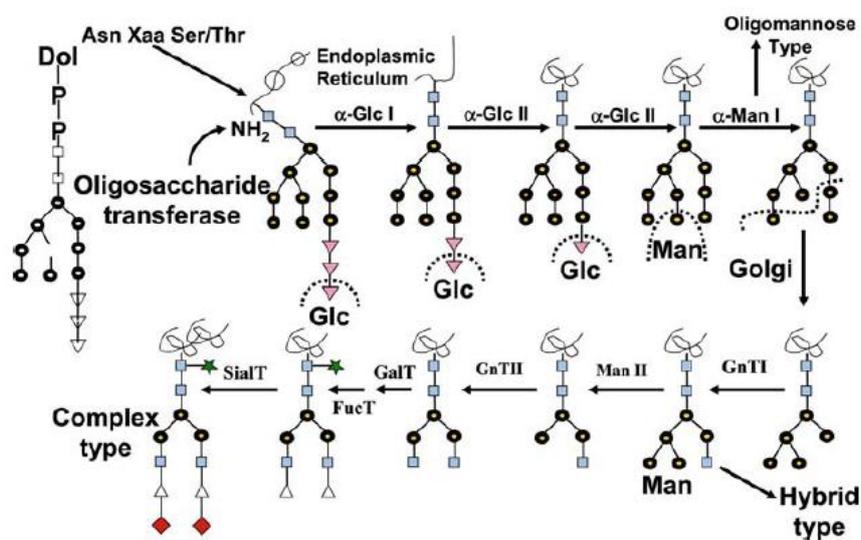


Figure 1.3.1.2.1: Glycosylation pathway for N-glycans (Butler, 2006).

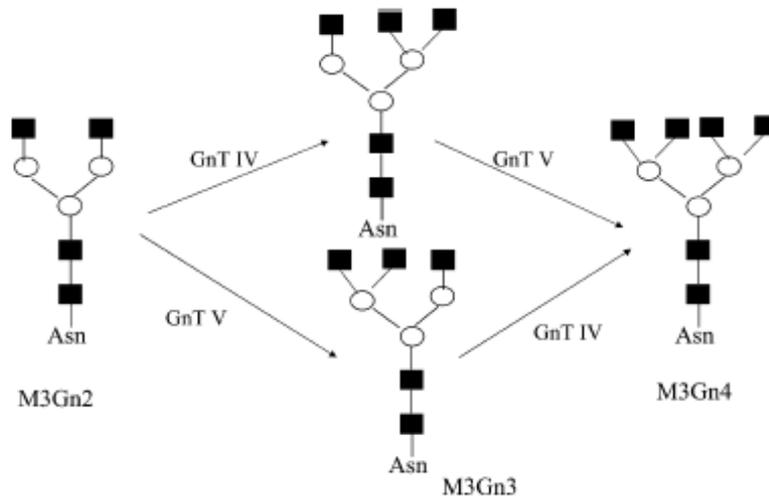


Figure 1.3.1.2.2: Production of tri- and tetra-antennary structure

1.3.1.3: O-linked glycans

O-linked glycans are smaller structurally than N-linked glycans and are added post-translationally to a fully folded protein (Butler, 2006). O-linked glycosylation differs to N-linked glycosylation in that it may be initiated in either the ER or the GA. N-linked glycosylation has been noted to begin in the ER only. (Hossler, 2009). The most common type of O-linked glycans are the mucin type. O-linked glycosylation of proteins begins with the transfer of N-acetylgalactosamine (GalNAc) from UDP-GalNAc to the hydroxyl group of a specific serine or threonine residue through an O-glycosidic bond. This reaction is catalysed by the enzyme GalNAc transferase (Jones et al., 2005). No consensus sequence for O-linked glycosylation has been identified. It has however been noted that it often occurs in regions of the protein with high proportions of serine, threonine and proline. These residues are thought to enable the region of the protein to assume a conformation that is accessible to the GalNAc transferase enzyme (Butler, 2005).

Initial attachment of the GalNAc residue to the folded protein is preceded with elongation of the glycan leading to a number of large structures. They are synthesised by various glycosyltransferases cumulating in 8 different core structures. These core structures may be further modified by sialylation, fucosylation, sulfation, methylation or acetylation. The most common core structure found on glycoproteins produced in CHO cells is the core 1 structure (Gal β 1-3GalNAc) (Butler, 2005).

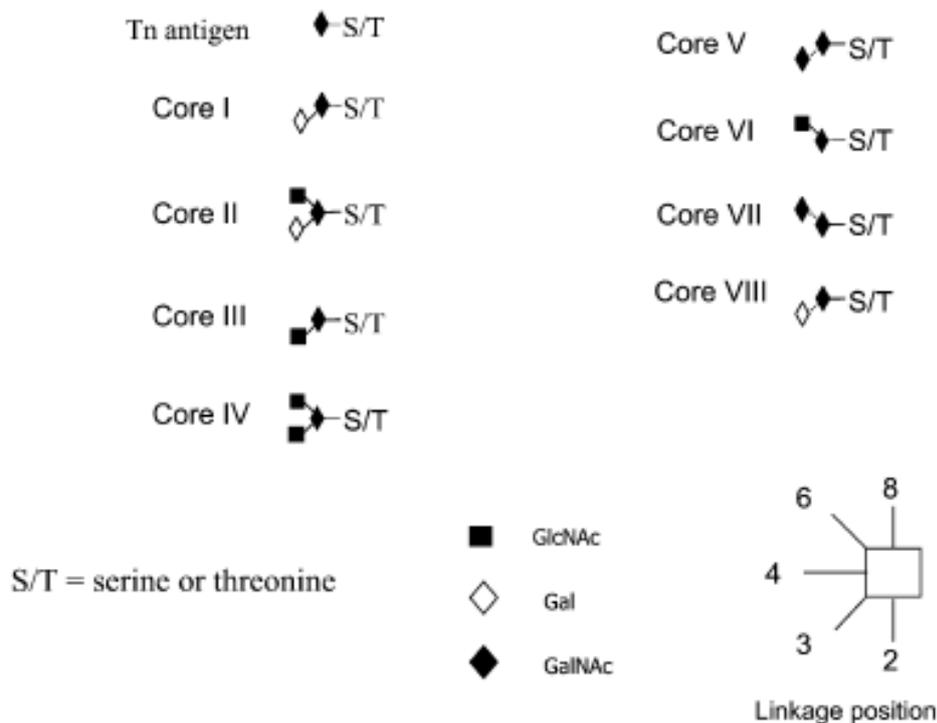


Figure 1.3.1.3.1: Processing of O-linked glycans in CHO cells (Butler, 2006).

1.3.2: *Biological properties of glycosylation*

Recent developments in functional glycomics have broadened our understanding as to the physiological function of specific carbohydrate groups attached to proteins (Butler, 2005). Several biological properties of recombinant therapeutic proteins rely on the possession of a specific glyco-form. Such properties include pharmacokinetics, secretion, in vivo clearance, solubility, receptor recognition and antigenicity (Hossler et al., 2009).

Bioactivity: Fucosylation is a key part of glycan processing in the GA, resulting in the transfer of a core α 1,6 fucose residue from the fucose donor GDP-fucose to the Asn-linked GlcNAc residue. However little is known regarding the function of this core fucose residue. It is understood that fucose plays a role in the removal of glycoproteins from the blood into the liver through the binding of fucose to receptors present on the surface of macrophage, initiating phagocytosis (Butler, 2005). However studies have shown that fucose has a key role in defining the oligosaccharide conformation needed to allow for protein-carbohydrate interactions. In immunoglobulin G1 (IgG1) α 1,6 fucose is important for antibody function as it determines the binding capacity of the protein to Fc receptors and is involved in antibody-dependant cell cytotoxicity (ADCC) (Hossler et al., 2009; Sethuraman and Stadheim, 2006). IgG-mediated ADCC can be modulated by varying the N-glycan composition. Also, during

glycan processing in the GA, GnT enzymes are responsible for the introduction of variable antennarity onto the core structure. The resulting N-glycan antennarity subsequently determines the level of terminal glycosylation which in turn may significantly impact the activity of the glycoprotein. E.g. In erythropoietin (EPO), it was specifically determined that tetrasialyated tetra-antennary N-glycans increased in vivo bioactivity in comparison to biantennary N-glycans (Hossler et al., 2009). N-glycan occupancy at a specific site on a glycoprotein has been demonstrated to essential for the activity of therapeutic proteins. t-PA has three potential glycosylation sites at Asn117, 184 and 448. Occupancy at Asn-184 modulates its binding affinity for lysine, a critical functional element in the activity of the protein (Jones et al., 2005).

Efficacy and pharmacokinetics: It has been reported that pharmacokinetic variability displayed by tumor necrosis factor alpha (TNF- α) antagonist is solely dependent on terminal GlcNac levels and independent of terminal galactose and sialic acid levels (Hossler et al., 2009). The number of N-glycan attachments to a recombinant therapeutic protein may also impact the efficacy of the protein. This has been demonstrated in EPO in which an increase in the number of N-glycan attachments resulted in an increase in the efficacy and in vivo activity of the protein (Jones et al., 2005).

Half-life and in vivo clearance: Complete glycosylation of recombinant proteins is usually associated with maximisation of galactosylation and sialylation. Often the processes are incomplete and this gives rise to considerable glycan structural variation (Butler, 2005). High levels of terminal sialylation of glycoproteins is important in order to avoid the affects of asialoglycoprotein receptors. These receptors are present in the liver and on the surface of macrophages and are responsible for the removal of glycoproteins from circulation, thus reducing their half life (Butler, 2006). The importance of high terminal sialylation has been noted however to vary between therapeutic proteins. Varying levels of terminal sialylation of monoclonal IgG1 antibodies have shown no significant differences in the clearance ratio (Hossler et al., 2009). However for non-antibody therapeutics such as EPO, the removal of sialic acid groups resulted in a significantly reduced half life (Butler, 2005).

The presence or absence of an N-glycan structure on a therapeutic protein may also affect the molecules half-life. E.g. N-glycan occupancy at Asn-117 on t-PA is known to affect its clearance from circulation. The addition of N-glycosylation sites to follicle stimulating hormone (FSH) increased the half life of the therapeutic protein (Jones et al., 2005).

Immunogenicity: Variations in the structure of sialic acid residues have been noted between species. N-glycolyl-neuraminic acid (NGNA) is found in goat, sheep and cows, whereas N-acetyl-neuraminic acid (NANA) is the sialic acid residue found in humans. The ratio of NANA to NGNA is dependent upon both the cell line and culture. NGNA is the predominant sialic acid found in mice. CHO cells however produce glycoproteins which typically contain NANA. However, up to 15 % of the sialic acid residues present on glycoproteins produced in CHO cells have been NGNA (Butler, 2006). The presence of high levels of NGNA on CT4-IgG fusion protein have been demonstrated to elicit an immune response (Hossler et al., 2009). Galactose monosaccharides have also been demonstrated to impact the immunogenicity of recombinant therapeutic proteins. α 1,3-linked galactose is known to be immunogenic (Butler, 2006). Incomplete processing of N-glycans may result in glycoproteins containing hybrid mannose structures which may elicit an immune response. In some cases this is a desired property and may be exploited for targeted drug delivery. E.g. Cerezyme, which is used in the treatment of Gaucher disease, has mannose sugars which facilitate macrophage recognition via the cell surface mannose receptors (Hossler et al., 2009).

1.3.3: Aggregation and glycosylation

Glycosylation plays a key role in determining the bioactivity of recombinant therapeutic proteins, primarily through the binding of the oligosaccharide residues to receptors at targeted areas, as described above. Effector function is not however the sole biological purpose for the existence of glycan structures on protein molecules. Sufficient evidence also exists suggesting that glycosylation has a stabilising effect on protein structure, thus reducing the likelihood of protein aggregation occurring (Pace, 1990; Karpusas et al., 1998; Sola and Griebenow, 2009).

Large molecule drugs offer a range of favourable properties as therapeutics such as higher target specificity and pharmacology potency, to name but a few, in comparison to the more conventional small molecule drugs which previously dominated the market. However one drawback of these macromolecular drugs is their higher levels of protein structure, required for therapeutic efficacy, which result in instability issues due to their noncovalent nature. Large molecule drugs display structural complexity and diversity that was never an issue with small molecule drug candidates. The challenge therefore lies in the long term stabilization of protein pharmaceuticals. Proteins exhibit only a marginal difference in thermodynamic stability between their folded and unfolded states. As a result of their colloidal nature,

proteins are prone to pH, temperature and concentration dependent precipitation, surface adsorption and non native supramolecular aggregation.

The instabilities of proteins are both chemical (proteolytic degradation, oxidation and chemical crosslinking) and physical (precipitation, chemical denaturation, thermal denaturation, kinetic inactivation and aggregation) in nature. Various strategies have been put in place to with the aim of overcoming the above instabilities and achieving long term stabilisation of protein pharmaceuticals. These include the use of stabilizing excipients (amino acids, sugars, polyols) and internal stabilisation by altering structural characteristics of the protein through chemical modifications e.g. mutations, glycosylation, pegylation (Sola and Griebenow, 2009).

The above strategies have all displayed a series of limitations in the stabilisation of protein therapeutics while maintaining complete effector functions of the drug molecule with the exception of glycosylation. It has been extensively reported that through manipulation of key glycosylation parameters including glycosylation degree, glycan size, and structural composition, the stability of the protein may be engineered as desired. Glycosylation has been demonstrated to curb all of the major chemical and physical instabilities in a variety of therapeutic proteins, as reviewed by Sola and Griebenow, (2009).

1.3.3.1: Chemical instabilities

Proteins are highly susceptible to proteolytic degradation, before they even reach their targeted areas. Proteolytic stability may be engineered into proteins through alteration of glycan structures. Enzymatic glycosylation of end-terminal glycan structures (GlcNAc, galactose and sialic acid) in IgG-like antibodies resulted in greater proteolytic stability in the presence of papain (Raju and Scallan, 2007). The presence of the glycans provided steric hindrance around the peptide backbone of amino acids adjacent to the glycosylation site thus conferring the protein with proteolytic stability. Oxidation of various amino acid side chains during storage of proteins has been associated with loss in bioactivity. The protein drug Erythropoietin (EPOGEN® Amgen, Ortho) is susceptible to tryptophan oxidation. Comparison of oxidative susceptibility between glycosylated and deglycosylated forms of the protein demonstrated that glycosylation diminished oxidation rates of the tryptophan (Sola and Griebenow, 2009). The existence of disulphide and non-disulphide crosslinking pathways in proteins allow for the formation of covalent dimers and oligomers, resulting in a loss of

bioactivity. Chemical glycosylation has been noted to suppress the self-association of protein into dimmers and oligomer species in insulin. (Baudy et al., 1995).

1.3.3.2: Physical instabilities

Protein stability is inversely proportional to its concentration. The specific concentration at which a protein will become unstable and precipitate out of solution is dependent upon the solution's pH, temperature, ionic strength, and excipient concentration. Glycosylation has been demonstrated to increase the solubility of many proteins. The solubility of the commercially available enzyme peroxidase has been shown to have a linear dependence with the degree of glycosylation. A logical explanation for such an observation would lie in the principle that glycans have a higher affinity for aqueous solvents than polypeptide chains. (Tams et al., 1999). However the increase in solubility of the protein is due to an overall greater molecular solvent accessible surface (SASA) caused by the presence of the glycans (Sola and Griebenow, 2009).

Environments of extreme pH disrupt internal electrostatic forces and charge-charge interactions in proteins leading to partial unfolding of the protein. This partial unfolding in turn causes a reduction in local charge density, followed by a further decrease in the electrostatic free energy of the protein which cumulates with global unfolding of the protein. Glycosylation has been documented to maintain conformational stability of Penicillin G acylase by up to 13 fold at pH 3 and 10 fold at pH 10 when compared to the unglycosylated form. Thermal denaturation of proteins has also been extensively studied. Coincidentally this is one of the most fundamental biophysical properties which becomes altered for proteins once they become glycosylated (Kim et al., 2004; Joao et al., 1992; Wormald and Dwek, 1999).

1.3.3.3: Protein aggregation

The tendency for proteins to form condensed phases (mainly amorphous aggregates) falls under the umbrella of physical instability issues affecting the vast majority of large molecule drug products (Sola and Griebenow, 2009). As with all other protein instabilities, glycosylation has been outlined as a promising solution to protein aggregation which is a significant problem during each stage of production and storage. Aggregation is responsible for decreases in yields at every stage of the production process including protein expression, upstream bioprocessing, purification, downstream processing and fill finish (Manning et al., 2010).

Protein aggregation is described as the condensation of proteins to form amorphous solids. These amorphous solids may exist as soluble or sub-visible aggregates in solution or phase separate and sediment to the bottom of the solution container thus being completely visible. Depending on the mechanism by which aggregates form, the aggregation may be reversible or irreversible (Philo and Arakawa, 2009). There are 5 main mechanisms by which aggregation may occur:

- (1) Self association: This process is characterised by the formation of protein aggregates even when the protein is in its native conformation and under thermodynamically favourable conditions without stress. This form of aggregation is reversible and its potential to occur may be measured by determining the osmotic second virial coefficient (B₂₂). Negative B₂₂ measurements indicate predominant protein-protein interaction potential, while positive measurements are indicative of protein-protein repulsion (Chi et al., 2003).
- (2) Aggregation through unfolding intermediates: Post-translational modification steps in protein synthesis involve the folding of protein in tertiary structures which acquire a stable conformation. However the process of protein folding may be incomplete and result in the presence of partially folded proteins. Hydrophobic patches on the surface of such proteins make them prone to aggregation (Fink, 1998).
- (3) Chemical modification: As described above, the macromolecule structure of proteins makes them susceptible to chemical instabilities such as oxidation, demerization, deamination, and hydrolysis. Such chemical modifications to proteins often promote the subsequent aggregation of the protein as they often result in changes in the hydrophobicity of proteins as well as their secondary and tertiary structures (Philo and Arakawa, 2009).
- (4) Nucleation dependent: The existence of preformed aggregates in protein solution reduces the overall energy barrier required nucleation and subsequently accelerates the process of aggregation. This process is referred to as homogeneous nucleation. The energy barrier may also be reduced by the presence of impurities or contaminants as oppose to preformed aggregates. This often results in heterogeneous nucleation (.Hamada and Dobson, 2002; Come et al., 1993).
- (5) Surface induced: Hydrophobic interactions between proteins and air-liquid interfaces and electrostatic interactions between proteins and surfaces of containers may result in aggregation of the protein in those specific areas (Manning et al., 2010).

1.3.3.4: Aggregation problems at upstream level

To date, therapeutic protein aggregation resulting from biopharmaceutical production processes has been considered a downstream processing issue (Manning et al., 2010), with the implications of upstream processing on aggregation being overlooked. The aggregation of proteins during downstream processing has been widely studied and extensively reviewed (Manning et al., 2010). However an examination of the full bioprocess of protein aggregation from cell culture to fill finish is essential. Protein aggregates may have a similar size and density as many other components in a cell culture environment including cell debris and also the cells themselves. As with all other chemical and physical instabilities noted for therapeutic proteins, various examples so exist in which protein aggregation has been prevented or induced due to the persistence of a specific glycoform.

Baudys et al., (1995) showed how the physical stability of insulin could be improved by reducing its aggregation kinetics through the chemical attachment of small sized glycans. The positive impact this had on aggregation was related to the transamidation crosslinking reaction which suggests a stabilising mechanism involving steric intermolecular repulsion phenomena. Ioannou et al., (1998) showed that glycosylation of α -galactosidase A at Asn 215 prevents aggregation by reducing the exposure of a surface hydrophobic patch on the protein. Similarly the deglycosylation of interferons promotes aggregation of the proteins due to the exposure of hydrophobic regions on the polypeptide chain. E.g. The primary structure of interferon- β (IFN- β) contains a high number of surface exposed hydrophobic residues (Phe-70, Phe-154, Trp-79, Trp-143), which are all in the vicinity of the glycosylation site. The large carbohydrate moiety of the glycans usually shields this area from the environment. Deglycosylation of the protein therefore exposes the hydrophobic residues, promoting aggregation of the protein. The deglycosylated form of the commercially available recombinant protein thyroid stimulating hormone (THYROGEN®: Genezyme) has also been demonstrated to be more prone to aggregation (Weintraub et al., 1983). Increased colloidal stability of the glycosylated form of phytase has also been observed (Hoiberg-Nielsen et al., 2006). This increased in stability was thought to be due to increased steric hindrance brought about by the attached glycan as oppose to being dependent upon the hydrophilic related properties of the glycans (Sola and Griebenow, 2009). The recombinant protein EPO has three N-linked glycosylation sites at Asn-24, Asn-38 and Asn-83 and one O-linked glycosylation site at Ser-126. The enzymatic or chemical deglycosylation of EPO was shown to induce aggregation of the protein (Dube et al., 1988). Kayser et al., (2011) compared the

stability and temperature induced aggregation of glycosylated and aglycosylated forms of human IgG1. It was demonstrated that at higher temperatures, the aglycosylated form of the IgG1 aggregated more rapidly than the glycosylated form.

All of the studies above suggest a mechanism in which protein aggregation is prevented due to the presence of a glycan on the protein surface (Sola and Griebenow, 2009). The size and heterogeneity of the glycan has also been demonstrated to play a significant role in the aggregation potential of a protein. Glycosylation is not a completely reproducible process and often results in a pool of different glycoforms of the protein being produced in a protein solution. Although glycosylation has been noted to have a stabilising effect on recombinant proteins, alterations in the glycan structure can be expected to alter both the solution and structural stability of the protein. Gomes and Herr, (2006) showed that aggregation of proteins could not be prevented by smaller sized glycans, independent of the amount of glycan attached to the protein. Human IgA has an O-linked glycosylation site in the hinge region. Undergalactosylation and undersialylation of this glycan structure have been linked to aggregation of the IgA molecule in a hinge specific manner. Undergalactosylation lead to increased adhesion of the molecule to extracellular matrix proteins.

Glycosylation has proven itself important for both bioactivity and effector function of recombinant therapeutic proteins, as described in, Section 1.3.2. The impact which glycosylation may have on the stability of the protein at both the upstream and downstream production levels give rise to the placement of further emphasis on the optimisation of culture conditions for efficient and correct glycosylation of recombinant proteins. Culture conditions which effect glycosylation, and in doing so which may affect the aggregation state of the protein have been investigated

1.3.4: Culture conditions affecting glycosylation.

The impact of various culture conditions on the growth and productivity of mammalian cell expression systems has been extensively researched. However as cells grow and produce recombinant proteins, the activities of the enzymes involved in the attachment of sugar residues onto emerging polypeptides, may also be influenced by the culture environment. Culture conditions affecting the glycosylation of recombinant proteins may in turn subsequently influence the bioactivity and in some cases the stability of the recombinant proteins.

The glycoform profile of recombinant glycoproteins has been reported in literature to be affected by various parameters including: the host cell line expressing the protein, the protein structure itself and the extracellular culture environment (Jenkins et al., 1996).

1.3.4.1: Host cell and protein structure

Choice of expression system for recombinant protein production include bacterial cells, yeast cells, plant cells, insects, transgenic animals and plants and mammalian cells such as rodent derived or human derived. One of the key requirements of any host cell line lies in it's ability to perform the post-translational modifications (including glycosylation) of the protein, thus enabling the protein to perform effectively as an FDA approved therapeutic. The above expression systems all vary in their abilities to perform glycosylation of therapeutic proteins and also to produce the correct glycoforms for therapeutic function, as reviewed by Jenkins et al., (1996).

Common bacterial expression systems such as *E. coli* have traditionally had no capacity to glycosylate proteins in neither N- or O-linked conformations. Lower eukaryotic cells such as yeast and fungi are typically associated with the production of hypermannosylation of recombinant proteins produced in such expression systems. The high mannose structures are typically released from the cells as end-products of glycosylation (Butler, 2006). The few studies which have been completed in plant cell systems have been primarily N-glycan structures lacking in sialic acid residues. Insect cells, another conventional host cell choice, are limited to produce only simple oligomannose-type oligosaccharides such as $\text{Man}_9\text{GlcNAc}_2$ (Jenkins et al., 1996). Mammalian cells exist as the host cells of choice for the commercial production of therapeutic proteins (Kim et al., 2012). Given that species which are mammals are phylogenetically closer to humans, it is expected that the glycosylation characteristics of recombinant proteins produced would be common to that of proteins produced by human cell lines and so increase the bioactivity of the potential drug candidate in humans through alleviating the possibility for immunogenic response.

However, as noted in Section 1.3.1, the entire process of glycosylation involves the transfer of oligosaccharide structures from nucleotide sugar donors onto the peptide backbone of newly synthesised polypeptide chains. These reactions are catalysed by a range of transferase enzymes present in the ER and GA of the cell producing the protein. This process is therefore not only influenced by the availability of a pool of nucleotide sugar donors in the cell, but also on the activity of a series of transferase enzymes. Variations in the activity of these enzymes across various mammalian cell lines have been responsible for variations in the

attached glycans onto protein. Some variations are independent of the conditions under which the host cell is grown (Butler, 2006).

The activity of GnT enzymes involved in the complex processing of high mannose intermediates into complex type glycans has been noted to vary in activity between mammalian cell lines. In particular GnT(III), the enzyme responsible for the transfer of β 1,4 bisecting GlcNAc residue, is absent in CHO cells. Glycoproteins produced in these cells usually lack β 1,4 bisecting GlcNAc (Butler, 2006). Sialylation of glycoproteins has been noted to be particularly varied between host cell lines. In section biological structures, two possible forms of sialylation (NANA and NGNA) found on mammalian cell proteins, and their importance in terms of effector function and immunological response were identified and discussed. NGNA levels have been shown to be more prevalent in recombinant proteins derived from mouse or human-mouse hybridoma (Jenkins et al., 1996). Most CHO cell lines used for recombinant protein production have an inactivated gene for α 1-3-galactosyltransferase and therefore make low levels of NGNA in comparison to NANA. However CHO cells have been noted to lack a functional α 2,6-sialyltransferase enzyme and so are solely involved in the synthesis of α 2,3-linked terminal sialic acid via α 2,3-sialyltransferase. Both sialyltransferase enzymes are present in mouse and human cell lines (Jenkins et al., 1996; Hossler et al., 2009).

The primary, secondary or tertiary structure of the recombinant protein is also an important factor affecting its glycosylation. The presence of a consensus sequence on an emerging polypeptide backbone does not guarantee the glycosylation of the protein. It is estimated that only approximately 65 % of all potential glycoprotein sequons become occupied by a glycan. Glycosylation proceeds folding of the protein and so it is proposed that often the inaccessibility of the sequon to the OST enzyme on the fully folded protein may be responsible for a lack of glycosylation of the protein (Butler, 2006). E.g. Di-sulphide bridge formation is a co-translational modification noted to cause inaccessibility of glycosylation enzymes. The elimination of a disulphide bond between Cysteine (Cys) 45 and Cys 51 in the protein interleukin-6 increases the efficiency with which Asn 46 is glycosylated (Weply, 1991). The position of a sequon is related to the primary structure of the protein. It has commonly been reported that the frequency of non-glycosylated sequons increases approximately 60 amino acid residues from the C-terminus (Opdenakker et al., 1993).

Related to both the host cell line and also the cultivation process parameters employed is the growth rate of the cells. As noted above, culture conditions allowing for high cellular growth

rates and high specific productivities are often employed. This may however have implications for the glycosylation of the produced recombinant protein. The growth rate of the cells and the rate of synthesis of the protein determine the extent of the time period in which the N-glycan is exposed to the OST enzyme. Site occupancy of a polypeptide chain is in fact dependent upon the elongation rate. The CHO produced recombinant protein t-PA has 3 N-glycan sites, one of which is variable at Asn-184. Anderson et al., (2000) showed that site occupancy at Asn-184 could be correlated with the fraction of cells in G0/G1 phase of the cell cycle. Furthermore, a temperature induced reduced growth rate of the t-PA producing CHO cell line resulted in increased site-occupancy at Asn-184. Therefore lowering the rate of protein translation improved glycosylation. In regards to the complex processing of intermediate glycan structures, residence time in the GA has also proven to be significant. Wang et al., 1991 demonstrated that it was possible to decrease the flow of glycoproteins through the GA by incubating the cells at 21 °C. The results were significant in that the GlcNAc content of the newly synthesised glycans increased by 100 %. In contrast however, Bulleid et al., (1992), showed that lowering t-PA synthesis in CHO cells had little effect on protein glycosylation.

1.3.4.2: Process conditions

Host cell line and cellular growth rate should be optimised in order to achieve the desired glycosylation profile of the recombinant protein for enhanced stability and bioactivity. Once the host cell line has been chosen, the cell culture conditions should be optimised in order to minimise glycoprotein heterogeneity and prevent deterioration of product quality, thus assuring the production of a certain range of glycoforms or even a specific glycoform (Jenkins et al., 1996). Normally changes in the glycosylation due to culture conditions are limited to the relative expression of glycoforms out of a pool of structures characteristic for a certain glycosylation site of a given protein. In order to maintain product quality it is essential to understand the parameters that cause variation in glycosylation macro- and micro-heterogeneity. The process conditions affecting the glycosylation of proteins are similar to those which affect other cellular activities such as growth and specific production rate.

1.3.4.2.1 Physical parameters

Biphasic cultivation methods employing temperature shift optimisation for enhanced cellular growth rate (phase I) and maximised specific productivities (phase II) are routinely

applied in recombinant protein bioprocessing (Fox et al., 2003). However changes in the temperature throughout the cultivation period may significantly affect the glycosylation profile of the recombinant protein produced. Trummer et al., (2006) demonstrated a reduction in the ratio of sialic acid to glycoprotein when the temperature was decreased from 37 °C to 30 °C for the CHO produced recombinant protein EPO-Fc. In the same study, a decrease in sialylation of 20 % and 44 % was noted when the temperature was reduced from the optimum to 33 °C and 30 °C respectively. Similarly, Rodriguez et al., (2010) also reported a lower degree of sialylation of β -IFN after the application of temperature shift from 37 °C to 32 °C in comparison to culture that remained at 37 °C. A decrease in glycan site occupancy was also reported for the temperature shift culture in comparison to the 37 °C culture. However other studies appear to contradict these findings on the effect of temperature shift on the degree of site-occupancy of glycoproteins. Anderson et al., (2000) reported an increase in N-glycan site-occupancy of t-PA when biphasic cultivation temperature shift from 37 °C to 32 °C was applied. Gawlizek et al., (2009) tested a broader range of temperatures for CHO cells producing the recombinant protein enzyme Glycoprotein 2 (GP2) and reported increased site occupancy of 2.3 % and 4 % when temperature shift to 33 °C and 31 °C was applied respectively. Moore et al., (1997) investigated the effect of temperature on nucleotide pools in CHO cell cultures. After the application of temperature shift from 37 °C to 30 °C, a sharp decrease in the pool of uridine tri-phosphate (UTP) was observed. A decrease in UTP as a result of changes in culture conditions is often coupled with an observed increase in the concentration of activated amino sugars such as UDP-GlcNAc and GalNAc, which this study reported. An increased ratio UDP-GlcNAc is thought to compete with the nucleotide sialic acid donor CMP-NANA for transport into the Golgi. It may therefore be proposed that the resulting decreases in the level of sialylation reported by Anderson et al., (2000) and Rodriguez et al., (2010) may be due to the increased concentration of UDP-GlcNAc in the GA at lower cultivation temperatures (Butler, 2006).

As stated in Section 1.2.2, There has been a limited amount of investigations to date as to the effect the pH of the external environment may have on cellular functions. It is considered that adverse external pH changes will in turn alter the pH of the Golgi. If the pH_i is altered outside the optimum range for the glycosylation enzymes, a decrease in activity and subsequent glycan processing may be observed (Butler, 2006). It is generally reported that a pH range of 6.9-8.2 does not have a dramatic effect on the glycosylation profile of recombinant proteins expressed in CHO cells. Underglycosylation outside of this range was

reported (Jenkins et al., 1996). Trummer et al., (2006) reported a maximum sialic acid to glycoprotein ratio at pH 7.0 for the CHO produced EPO-Fc. This ratio was noted to decrease when conditions were altered above or below this pH. The external pH of the culture environment was also noted to have implications for N-glycan site-occupancy. Gawlitzek et al., (2009) showed a decrease in N-glycan site occupancy of the recombinant protein enzyme GP-2 with increasing external pH from 7.05 to 7.15. A similar trend was also noted for the level of terminal sialylation of the N-glycan. It was deduced that the level of terminal sialylation could be related to site-occupancy. Higher site-occupancy provided increased N-glycan substrate for the 2,3 sialyltransferase to attach sialic acid residues to the terminal galactose residue.

One of the main components of a cells culture environment is the medium in which they grow. Due to the fact that over 30 essential nutrients are necessary for cell survival and that the requirements of the cell are very much dependent on cell type and process conditions, many different forms of culture media have become commercially available. The variation in the concentrations of nutrients in such media does in itself have extensive implications on cell growth, productivity and importantly recombinant protein quality. Irrespective of nutrient concentration, the osmolality of the medium has also been demonstrated to affect the glycosylation profile of recombinant proteins. As noted above in Section 1.2.3 cell culture medium is typically designed to have an osmolality between 260-320 mOsm/kg (Ozturk and Palsson, 1991). However throughout the culture period, the osmolality of the medium may change, e.g. through the addition of base (NaOH) for pH control. Pacis et al., (2011) conducted studies using a CHO cell line producing the recombinant protein IgG1 which proved indicative of decreased glycan processing at osmolalities outside of the acceptable ranges. Increased osmolality of 1250 mOsm/kg resulted in a 23-27 % increase in the level of intermediate Man5 glycoforms in comparison to the 750 mOsm/kg control culture. The increase in Man5 glycoforms was related to a decrease noted in complex galactosylated glycoforms at the elevated osmolality level. Other cell lines followed a similar trend but with different absolute levels of Man5.

The detrimental effects of increased CO₂ levels in culture coupled with increases in medium osmolalities have been discussed above, also in Section 1.2.3. The glycosylation profile of recombinant protein may also be altered at elevated pCO₂ levels. Kimura et al., (1997) investigated the glycosylation profile of the CHO produced recombinant protein t-PA at an elevated pCO₂ concentration of 250 mmHg at medium osmolality levels of 210 and 376

mOsm/kg. No changes in the sialic acid content or the content of other monosaccharide (mannose, galactose, and fructose) were found for the glycoprotein. However the % of NGNA in comparison to NANA was lower 60 % lower at 250 mmHg pCO₂ in comparison to control cultures. Two mechanisms were proposed for these findings. The first coincides with a reduction in the activity of the enzyme CMP-NANA-hydroxylase which is responsible for the conversion of CMP-NANA to CMP-NGNA in the cytosol. It was predicted that alterations in the pHi of the cell brought about by the increased pCO₂ levels affected activity of the enzyme. CO₂ is nonpolar and diffuses across the cell membrane where it hydrates and dissociates into H⁺ and HCO₃⁻ ions causing increasing in the pHi of the cell. The cell attempts to counteract this effect by increased activity of Na⁺/H⁺ and/or HCO₃⁻/Cl⁻ antiporters. Such activities do not however assure that the pHi returns to its original value, thus affecting the activity of a range of glycosylation enzymes such as CMP-NANA-hydroxylase. The second mechanism proposed for the reduction in CMP-NGNA is related to alterations in cellular metabolism brought about by elevated pCO₂ levels. pCO₂ may affect NADH supply through affecting glucose or glutamine consumption or oxygen consumption. NADH is utilised in the biosynthesis of CMP-NGNA.

In relation to the concentration of dissolved gases present in cell culture medium, the effect which the level of DO has on the glycosylation profile of recombinant therapeutic proteins has been extensively reviewed. Varying results have been published suggesting that the effect of DO on the glycosylation of recombinant proteins varies between cell lines and the protein produced and may also depend on the cell culture technique employed, amongst other variables (Restelli and Butler 2002). It may be recalled from Section 1.2.4, that Normal or normoxic conditions exist when 100 % air saturation exists. Under mild hypoxia conditions Lin et al., (1993) showed that a minimal effect on the glycosylation of CHO produced t-PA. However similar conditions were demonstrated to increase the levels of sialylation of FSH also produced by CHO cells (Choitigeat et al., 1994). In such studies it was deduced that the increases in the level of sialylation were due to increases in the activity of the sialyltransferase enzyme. In contrast to this report, Trummer et al., (2006) documented that the maximum sialic acid to glycoprotein ratio may be observed at 50 % air saturation for the recombinant protein EPO-Fc produced in CHO cells. The level of DO has also been noted to have implications on the galactosylation of recombinant proteins. Kunkel et al., 1998 reported a decrease in digalactosylated glycans from 30 % at high DO levels to 12 % at low DO levels. In a similar study a reduction in the level of galactosylation of N-glycan chains at lower DO

levels was reported for a murine hybridoma cell line producing mAb (Kunkel et al., 2007). This was denoted by increases in the relative amounts of agalactosyl chains and a decrease in the relative amounts of digalactosyl chains. Various mechanisms by which galactosylation may be affected by the DO level of the culture environment have been proposed. One of which suggests that varying levels of DO may reduce the availability of uridine di-phosphate galactose (UDP-Gal) due to reduced transportation in the lumen. The second mechanism proposed is based on the principle that low DO levels may affect the redox environment in the golgi which in turn alters the pathway for inter-chain disulphide bond formation on recombinant proteins. The altered pathway may be associated with early formation of such inter-heavy chain disulphide bonds. A relationship has been noted between the level of Fc galactosylation and the timing of formation of the disulphide bonds (Rademacher et al., 1996). The fucosylation of N-glycan structures attached to recombinant proteins has been illustrated to be effected by the level of DO in the culture environment. Restelli et al., (2006) observed that maximum core-fucosylation (80%) of recombinant human EPO produced in CHO cells occurred at 50 % DO. Higher or lower DO resulted in reduced fucosylation. The decreased fucosylation at such extreme levels was related to decreased activity of the Golgi enzyme α 1,6 fucosyltransferase.

As noted in Section 1.2.4, the level of DO in a culture environment is very much dependent upon the balance between the OTR and the OUR displayed by the cells. However irrelevant of this balance, a gradient in the level of DO throughout the bioreactor environment may be observed which is solely based on reactor design and mixing. Zhang et al., (2010) recently reviewed the expected gradients in the level of DO in a 8m³ scale reactor with a 2/0 rush ton turbine. Dependent on the OUR and mixing times, differences in oxygen concentration throughout the bioreactor may be seen resulting in non-consistent dissolved oxygen tension (DOT). Serrato et al., 2004 (Scaling 5) investigated the effect of such oscillations in DOT, in the range of 800-12800, on hybridomas producing mAbs. The main finding portrayed that mAb produced under the oscillating conditions had a higher amount of triantennary and sialyated glycans. Increasing the oscillation period to 12,800 s decreased the percentage of ungalactosylated glycans and also glycans containing only one galactose unit by 14 %. Glycans containing 3 galactose residues and sialyated glycans increased by 23 %. It was deduced that the oscillations did change the redox potential of the cell and also affected the folding of the mAb protein which in turn resulted in increased galactosylation. The result do to some extent contradict the predictions proposed by Rademacher et al., (1996) in that

galactosylation was increased verifying the initial statement that results are cell line and recombinant protein dependent.

In relation to bioreactor design and mixing, shear stress created through agitation and aeration have been noted to impact on the glycosylation of recombinant proteins, specifically site-occupancy. Senger and Karim, (2003) reported that maximum levels of damaging shear stress were required in order to reduce the extent of Asn-184 site occupancy in t-PA produced from CHO cells. Under conditions of shear stress it was presumed that the protein spent less time in the ER, resulting in the reduced attachment of glycostructures. Comparisons have also been made between the glycosylation profiles of recombinant proteins produced in different culture vessels. Wang et al., (2002) demonstrated variable terminal sialylation of N-glycans present on recombinant EPO produced from CHO cells cultivated in T-flasks, stirred tank bioreactors and cytopilot fluidised bed bioreactors. The effect of various culture conditions outlines above may also vary depending on the cultivation vessel. Kunkel et al., 2000 saw a difference in the effect of DO on the galactosylation of mAb produced from a murine hybridoma cell line in two different bioreactors, the LH series 210 (LH) and the New Brunswick Scientific (NBS) CelliGen. The reduction in galactosylation at high DO was noted to be less pronounced in NBS in comparison to LH. mAb proteins harvested from the NBS bioreactor contained higher levels of sialylated glycans in comparison to those harvested from the LH reactor. The differences in glycosylation patterns were explained by differences in oxygen supply and control strategies between the two bioreactors.

1.3.4.2.2 Chemical parameters

The environmental culture parameters noted to impact on the glycosylation profile of the recombinant therapeutic protein may be described as physical cultural conditions. The most important chemical cultural environment is the media in which the cells are cultured. Cellular growth and productivity is affected by the depletion of a number of nutrients from the culture media and the subsequent release of metabolic by-products into the media over the course of the culture period. The most predominant nutrients which have been noted to impact on the quality of the recombinant proteins are glucose, glutamine and their corresponding by-products, lactate and ammonia.

It has been reported for some time that glucose starvation decreases glycosylation efficiency (Baumann and Jahreis 1983, Chapman and Calhoun 1988) resulting in alterations in the degrees of site-occupancy of glycans on recombinant proteins. Hayter et al., (1992) illustrated that glucose deprivation resulted in the increased production of non-glycosylated gamma-

interferon. A number of studies have been completed investigating the resulting decreases in glycosylation efficiency. Glucose starvation may result in an intracellular depletion or a shortage of glucose-derived precursors of glycans. This concept was first proposed by Rearick et al., (1981). It was illustrated that under glucose deprived conditions, CHO cells rapidly cease to synthesize the 14-mer oligosaccharide glycosylation precursor $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ and alternatively accumulate $\text{Man}_5\text{GlcNAc}_2$ which is subsequently glucosylated and transferred onto the polypeptide backbone. The shift from synthesis of $\text{Man}_9\text{GlcNAc}_2$ to $\text{Man}_5\text{GlcNAc}_2$ was thought to be due to a decrease in cellular Dol-P-Man as the first 5 mannose residues in the oligosaccharide come from GDP-mannose and the last 4 come from Dol-P-Man. It was concluded that the enzyme that adds the 6th mannose residue is very sensitive to small changes in Dol-P-Man concentration. In this case glycosylation did still occur, through a different pathway. This study does therefore not propose a mechanism as to how site-occupancy is affected by glucose starvation. Nyberg et al., (1999) proposed that reduced site-occupancy under glucose starvation conditions was due to a decrease in the synthesis of UTP, the precursor for UDP-GlcNAc. It was hypothesised that during glucose limitation, available carbon is preferentially utilised for energy production and subsequently leads to reduced nucleotide biosynthesis. Under conditions of glucose starvation the declines in nucleoside triphosphates such as UTP could not be explained by increases in mono- and diphosphates as all nucleosides were being depleted. It was proposed that the nucleosides were continually consumed for RNA synthesis under conditions of glucose starvation. This concept was proven through the inhibition of protein and RNA synthesis, by which it was observed that the decline in nucleosides was alleviated. Lower levels of nucleoside triphosphate pools in turn cause lower levels of nucleotide sugar pools resulting in lower levels of glycosylation site occupancy. The results presented above so however again appear to be cell line and recombinant protein specific. Wong et al., (2004) found that in cases of glucose and glutamine limitation, only 6-9% of the recombinant protein IFN- γ was unglycosylated. It was concluded that extreme starvation was required in order for a significant impact on site-occupancy to occur.

Wong et al., (2004) also investigated the effect of glucose limitation on the microheterogeneity of glycan structures attached to Asn-25 and Asn-97 of IFN- γ . Glucose deprivation in the presence of glutamine control affected the complete processing of high-mannose types to full complex glycans resulting in a greater number of hybrid type glycans at both sites. Similarly to above, it was proposed that the presence of the high mannose type

glycan was due to limitations in the availability of uridine di-phosphate N-acetylhexosamine (UDP-GlcNAc). The elimination of glucose deprivation may be achieved through the implication of fed-batch mode of cultivation. However in order to define feeding strategies it is necessary to determine the critical glucose concentration at which glycosylation is affected. Xie et al., (1997) compared fed-batch cultures containing initial glucose concentrations of 2.25 mM with batch cultures in which the initial glucose concentration was 20 mM. Dynamic glycosylation efficiency was noted to be effected when the glucose concentration reached 0.6 mM which was indicated by a 67 % reduction in glycosylated species present. Glycosylation efficiency was however recovered when the glucose concentration was brought back to 2.34 mM. The results therefore indicate that exposure to low glucose concentrations for a short period of time does not permanently damage the glycosylation machinery.

Glutamine is an amino acid which is utilised by cells that have high energy demands and synthesise large amounts of protein and nucleic acids in favour or in conjunction with glucose as a carbon energy source. The metabolic fates of glutamine in mammalian cells have been extensively documented and reviewed (Vriezen et al., 1997 and Schneider et al., 1996). Typical concentrations of glutamine in mammalian cell cultures vary in the range of 1-7 mM (Freshney, 1987). Starvation of glutamine brought about by a limitation in the culture media has also been noted to have implications for the glycosylation of produced recombinant proteins. Glutamine starvation has been proposed to result in a decrease in the galactosylation of glycan structures on a newly emerging polypeptide chain. Nyberg et al., (1999) provided a mechanism by which this may occur. During glutamine starvation, amino sugar formation limits UDP-GNac synthesis, since glutamine is the ammonia donor in this reaction. The glutamine;fructose-6-P amidotransferase (GFAT) enzyme responsible for amino sugar formation transfers an ammonia group from glutamine to fructose-6-P to form glucosamine-6-P. The rate of the reaction depends upon glutamine conc. Limited glutamine supply for amino sugar formation can explain low UDP-GNac. Ryll et al., (1994) explained why feeding with amino sugars such as glucosamine or galactosamine leads to accumulation of UDP-GlcNAc. The excess amino sugars are converted into hexosamine phosphates, acetylated, and reacted with UTP to make UTPGlcNAc. As the GFAT reaction is not reversible, the majority of hexosamine formed accumulates as nucleotide sugars. Glutamine limitation in cultures has also been associated with the prevalence of high mannose structures and reduced terminal sialylation. Wong et al., (2004) studies the effect of glutamine and glucose limitation and feeding on glycan microheterogeneity. Microheterogeneity of glycans

at Asn25 and Asn97 of IFN- γ were relatively unaffected by glutamine limitation. They did however see an increase in hybrid types at both glycosylation sites and one extra high mannose type at Asn25 that were not present in batch cultures where glutamine was not limited. Such results illustrate that glutamine limitation can affect complete processing of high-mannose types to full complex types resulting in hybrid types. Additional glucose control resulted in a greater number of high-mannose type glycans at both glycan sites. It was concluded that in all cases the presence of high mannose types were due to limitations of UDP-GlcNAc caused by limited glutamine and glucose. The effect of glutamine limitation on terminal sialylation was also investigated. Limiting the glutamine concentration to 0.5 mM in control cultures, in comparison to a concentration of 4 mM in batch cultures, did not affect sialylation. However at concentrations of 0.3 mM and 0.1 mM sialylation decreased by 17 and 23 % respectively. It was proposed that this was due to impaired sialyltransferase activity or a low concentration of substrate or donor CMP-NANA. Also UDP-GlcNAc is essential for formation of ManNAc which is a precursor of CMP-NANA. UDP-GlcNAc has been well documented to be reduced in glutamine and glucose limited cultures.

Typically yield concentrations up to 5 mM ammonia are expected for typical concentrations of glutamine added to cultures (Hayter et al., 1991). The impact of increasing ammonia concentrations in culture on recombinant protein glycosylation has also been investigated. The overall effect of ammonium ions on recombinant protein glycosylation include increases in glycan antennary (Gawlitzeck et al., 1998) and reduced levels of sialylated glycans (Hossler et al., 2009; Jenkins et al., 1996). Overall, two main mechanisms for the inhibition of sialylation by ammonia have been proposed. The first mechanism outlines how ammonium ions induce increased glycan antennary and decreased sialylation. Enhanced incorporation of ammonia into glucosamine increases the ratio of UDP-GlcNAc/UTP ratio. Glucosamine is a precursor for UDP-GlcNAc. This mechanism has been described for a number of cell lines including BHK, CHO, Ltk929 and hybridoma and contributes to the formation of more complex glycans through the transfer of glucosamine and galactose units from the increased concentration of nucleotide donors, onto the glycan chain (Gawlitzeck et al., 1998). In the presence of 30 mM NH₄Cl, the UDP-GNAc pool accounted for 60 % of the total nucleotide pool in comparison to 9.2 % for standard culture conditions (Yang and Butler, 2002). Although increased pools of UDP-GNAc describe the effect of ammonia on increased glycan antennary, it has also been suggested as a plausible mechanism for the decreased sialylation observed under high ammonia concentrations. Pels Rijcken et al., (1995) described how

increased pools of UDP-GNAc and their subsequent increased transfer into the trans-golgi may impair the transport of Cytidine 5' -monophospho-N-acetyl neuraminic acid (CMP-NANA) into the golgi resulting in decreased sialylation due to lack of available sialic acid donors. This nucleotide sugar competes with CMP-NANA for transport into the golgi and therefore decrease the amount of CMP-NANA available for sialylation. The second mechanism suggests that ammonia raises the pH of the golgi thereby shifting the optimal pH of the sialy transferase enzymes (Butler, 2006; Hossler et al., 2009; Jenkins et al., 1996). Ammonium ions act as a weak base, which accumulate in the slightly acidic trans-golgi compartment, altering the pH outside of the range for optimal sialy transferase activity (Anderson and Goochee, 1995; Gawlitzek et al., 2000). This concept is supported by the findings of Barasch et al., (1991) in which it was reported that defective acidification of intracellular organelles in the cells of cystic fibrosis patients resulted in reduced sialylation of glycoproteins and lipids brought about by reduced sialytransferase activity. A pH of 7.0 in the trans golgi results in a 2-fold decrease in the activity of these enzymes (Anderson and Goochee, 1995). Jenkins et al., (1996) reported increasing ammonium ion concentrations, above 2 mM, may compromise sialytransferase present in GA. This resulted in reduced α 2,6-linked sialic acid in G-CSF produced in CHO. In the study increases in ammonium ions also reduced extent of recombinant placental lactogen N-glycosylation by CHO cells, but was dependent on pH. The interaction of ammonia on glycosylation genes has also been more recently investigated as a plausible mechanism for alterations in glycosylation at increased ammonia concentrations (Chen and Harcum, 2006).

Although it was stated above that the most important medium components to affect the glycosylation profile of recombinant protein are glucose and glutamine, a significant amount of research has also been completed assessing the effect of other media components on protein glycosylation. The inclusion of sodium butyrate in culture media has been associated with increasing the specific productivity of the cells while decreasing the cellular growth rate by up regulating apoptosis mechanisms (Hossler et al., 2009). Although the inclusion of the small molecule medium component has beneficial effects on growth and productivity, studies conducted by Sung et al., (2004) suggest that an overall decline in product quality is to be expected. CHO DUKX-B11 cultured in shake flasks under varying concentrations of sodium butyrate resulted in a product displaying increased microheterogeneity and reduced levels of sialylation. Other studies have assessed the effect of medium fortification with amino acids on recombinant protein quality. Crowell et al., 2007 reported an increase in the level of

sialylation by supplementing with amino acids which had been depleted from the culture at earlier stages. The inclusion of nucleoside-sugar precursors such as glucosamine has also been investigated as these precursors affect the intracellular nucleotide sugar pools and the subsequent resulting sialylation and antennary levels. Yang and Butler, (2002) demonstrated that the incubation of recombinant EPO producing CHO-K1 cells in a concentration greater than 10 mM glucosamine resulted in a 37 % decrease in the sialylation on tetrasialyated glycans. It was determined that the intracellular pool of UDP-GlcNAc was at least 2-fold higher due to glucosamine addition. As explained above, the resulting decrease in terminal sialylation may be due to the decreased pool of CMP-NANA resulting from increased levels of UDP-GlcNAc. As well as influencing the nucleotide sugar pools, many other medium components can influence the activities of the glycosyltransferase enzymes. Manganese for example is a metal ion cofactor of the GnT enzymes. Pacis et al., (2011) reported an ~ 10 % decrease in the level of intermediate high mannose structures on recombinant IgG1 upon supplementation of the media with 1.0 μ M $MnCl_2$. It was deduced that the addition of appropriate concentrations of $MnCl_2$ to culture media, in the range of 0.25-1.0 μ M may be beneficial in reducing undesirably high levels of high mannose hybrid glycans and achieving more complex glycans as a result of GlcNAc addition by GnT enzymes. Gawlitzek et al., (2009) tested the effect of additional manganese in the range of 10 nM – 100 μ M for increased site occupancy of N-glycans on t-PA. Site occupancy was increased by 2.5 % in comparison to other cultures. . It was proposed that the increased site occupancy was as a result of increased OST activity resulting from the manganese. No specific changes in site occupancy were noted between 100 nM and 100 μ M.

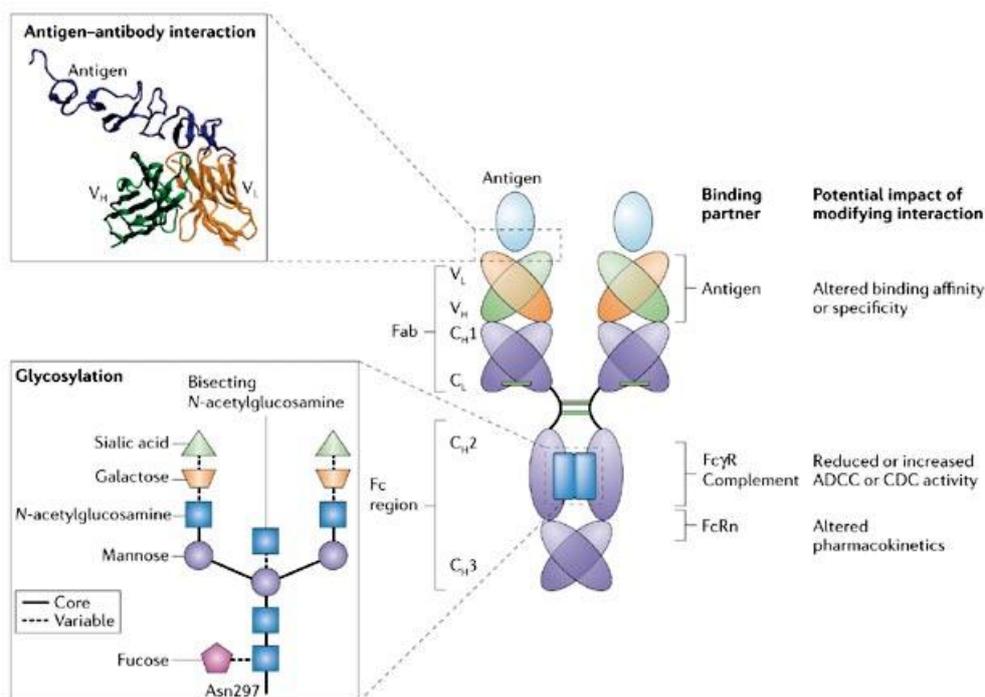
1.4: Glycosylation of rIgG1

The basic structure of an IgG molecule is of four polypeptide chains, two heavy chains (molecular weight 50 kDa) and two light chains (molecular weight 25 kDa). The heavy chain has one variable region and three constant regions. The light chain has one variable region and one constant region. The variable regions are responsible for antigen binding (Conroy et al., 2009). Through the formation of covalent (16 for IgG1 and IgG4, 8 for IgG2) and noncovalent bonds, the two heavy and light chains form three independent protein moieties connected through a flexible linker known as the hinge region (Beck et al., 2008). Two of these regions are known as the Fab regions. They are identical and contain the variable, antigen binding, regions. The third region is referred to as the Fc region is a

homodimer and consists of three domains; C_{H1}, C_{H2} and C_{H3}. The C_{H2} domains have a highly conserved N-glycosylation site at asparagine-297 (Asn-297) (Jefferis 2005). IgG Fc N glycans are usually biantennary complex type structures and usually contain a conserved heptasccharide core composed of 2 GlcNAc, 3 mannose and 2 GlcNAc residues that are β -1,2 linked to α -6 mannose and α -3 mannose, forming 2 arms (Beck et al., 2008).

Most of them are core-fucosylated and are characterised by low levels of galactosylation and sialylation. The largest variation in the glycosylation of rIgG1 has been noted in the level of galactosylation with biantennary core fucosylated structures containing, two, one or no galactose residues (Huhn et al., 2009). The rIgG1 glycoform produced by CHO cells can vary widely from clone to clone and are also very much dependent upon cultivation conditions. IgG antibodies produced in mammalian cells generally contain low levels of high mannose Glycoforms (Man5-9), because most glycoproteins are processed to complex Glycoforms G0F, G1F, and G2F. (van Berkel et al., 2009).

Cell lines producing rIgG1 should be selected based on the production of the IgG with fucosylation, galactosylation, and/or sialylation depending on the desired effector mechanisms, containing minimal levels of oligomannose-type glycans and putative antigenic and/or immunogenic glycan structures (van Berkel et al., 2009).



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Figure 1.4.1: IgG1 structure containing one consensus N-linked glycosylation site as Asn297.

1.5: Objectives of the PhD thesis

The demand for increased yields in recombinant therapeutic proteins from mammalian cell culture systems is constantly rising, requiring cheaper, more efficient, cost effective process optimisations than those which currently exist. The biotechnological industry has responded to this need with advances being made in cultivation systems and their capacities, allowing for the upscaling of mammalian cell cultures resulting in reported increased volumetric productivities of 100-fold over the past decade (Jain and Kumar, (2008). Scale-up of cultures to large volumes in continuous stirred-tank reactors requires continuous monitoring and control of various parameters such as temperature, pH, concentration of dissolved gases etc, which have been noted in the above review to affect cellular activities across all scales. With the introduction of the Process Analytical Technology (PAT) framework initiative by the Food and Drugs Authority (FDA), in 2001, measures have been outlined for the strict monitoring and control of the above process parameters. PAT may be

defined as “a system for designing, analysing, and controlling manufacture through timely measurements (i.e. during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of insuring final product quality” (Food and Drug Administration 2009). This initiative outlines the requirement for a more efficient online monitoring system of the entire production process with the key term “quality by design” coming to the fore. Therefore in seeking FDA approval for recombinant proteins as therapeutic drugs, it is first necessary to identify the CPP’s affecting the mammalian cells in culture, with particular attention being paid to the CQA of the recombinant protein produced. Once such CPP’s have been identified, monitoring and control systems may be designed and put in place enabling the entire production process as well as the product quality to be monitored throughout the entire production process as oppose to at the end of the process (Lopes et al., 2004).

Taking into account the ever increasing demand for higher cell and recombinant protein yields and also emphasising the strict necessity for monitoring and controlling recombinant protein quality, a project was designed entitled “High density CHO cell cultures: improved productivity and product quality”. The project was designed with the following aims and objectives.

Aim 1: Determination of the effect of ammonia, L-glutamine and L-glutamine substitutes on rIgG1 glycosylation and aggregation

Background: One of the CPP’s affecting the quality of recombinant proteins is the metabolic profile of the cells in culture. Nutrients, such as glutamine not only provide the energy for cell growth but are also necessary for the synthesis of nucleotide sugar donors which act as precursors for efficient glycosylation of the produced proteins. The metabolism of nutrient components such as glutamine results in the production of metabolic waste products which effect cell growth and productivity. The quality of the protein may in turn be outwardly affected by changes in cell growth and productivity rates. There is also cause for concern that the presence of metabolic by-products also affects protein glycosylation. It has been reported that the inefficient energy metabolism of mammalian cells in culture is still a major limiting factor for improvements in large scale high cell densities and recombinant protein production. The PAT initiative has also placed focus on the necessity to monitor and control nutrients as CPP’s in mammalian cell processes. Extensive research in PAT has focused on the

development of tools for monitoring and controlling such metabolites in cell culture, including infrared spectroscopy (IR) (Foley et al., 2012). Therefore in an initial investigation into the progression towards high density CHO DP-12 cell cultures it was first necessary to define the window with which cell growth, productivity and product quality could potentially be optimised through the alteration of critical media components such as l-glutamine and ammonia.

Objective: Varying the concentration of L-glutamine in the culture media will enable the determination of the suitable L-glutamine concentration required for achieving high cell densities using the above cell line. In addition to high cell densities, product yield and quality will also be critical factors used for determining the ideal concentration. While varying the concentration of L-glutamine, it is expected that a range of concentrations of ammonia will accumulate in the different cultures. The impact of an accumulation of specific ammonia concentrations on cellular activities may also be indirectly investigated. L-glutamate will also be investigated as a possible carbon source allowing for cell growth and productivity of this cell line, in the presence of reduced ammonia concentrations. The experiments will be conducted at small scale in a 1 L shake flask platform, allowing for efficient identification of suitable L-glutamine concentrations for optimised cell and product yields. The majority of research conducted to date investigating the effect of ammonia on protein quality (i.e. glycosylation) has involved the introduction of varying ammonia concentrations at the beginning of the culture period. The effects on protein glycosylation have been primarily concerned with the effects of ammonia on terminal sialylation, i.e. the last step of IgG1 glycosylation. Through a collaboration with the NIBRT based Dublin-Oxford Glycobiology group, a range of N-glycan structures may be identified from rIgG1 samples harvested from cultures. Therefore a more dynamic outlook of the effects of ammonia on the glycosylation profile of rIgG1 produced in the CHO DP-12 cell line may be obtained. As previous studies conducted investigations through the means of high initial ammonia concentrations, it is impossible to determine if the reports regarding the affect of ammonia on glycosylation do to some extent over-estimate the impact. Another objective of this study is to therefore to determine if the effect of ammonia on glycosylation may be different when high ammonia concentrations accumulate as a result of cellular metabolism or chemical breakdown of L-glutamine in comparison to when high initial ammonia concentrations are added to the culture. A range of ammonia concentrations will be added initially to cultures. The effect

such additions have on cellular activities may be compared and contrasted to those L-glutamine cultures which gave rise to similar ammonia concentrations by means of accumulation. The aggregation state of the protein harvested from all of the cultures will be identified. Any alterations in the aggregated state will be compared to alterations in the heterogeneity of the glycosylation of the protein with the hope in identifying a link between the presence of a specific glycoform and aggregation of the protein.

Aim 2: Scale-up of shake flask cultures to 1.7 L minifors reactor: Investigation of alterations in metabolism of rIgG1 glycosylation and or aggregation.

Background: In order to enhance cellular yields and volumetric productivities in mammalian cell cultures, the biotechnological sector is continuously developing new methods for the scale-up of cultures. The continuing platform of choice for industrial production of recombinant proteins from mammalian cell lines is the stirred tank bioreactor. Although scale-up does provide a mechanism for the enhancement of volumetric productivities, in comparison to typical yields achieved at small scale in shake flask platforms, the phenomenon of shearing brought about by mechanical agitation and sparging for aeration can have detrimental effects on cell and product yields.

Objectives: Once the optimal L-glutamine concentration for optimised cell yields, productivity and product quality have been identified at small scale, it is necessary to scale-up such cultures to a bench top stirred tank reactor platform. A dynamic analysis of the effects of scale-up on all cellular activities such as growth, metabolism, productivity and glycosylation, will be carried out. Any alterations in the cellular glycosylation profile of the rIgG1 from scaled-up cultures in comparison to shake flask cultures may be linked to alterations in the activities of the cells due to the influence of shearing brought about by mechanical agitations and aeration through sparging. Aggregation analysis will also be completed on the rIgG1 harvested from the minifors reactor cultures with the aim of identifying an effect of scale-up and/or glycan heterogeneity on aggregation of the protein.

Aim 3: Encapsulation of CHO-DP12 cells in alginate-poly-L-lysine-alginate microcapsules: Implications on rIgG1 quality.

Background: The CHO DP-12 cell line used in this project typically grows in suspension. High cell densities in suspension cultures are however impeded by mechanical and shear forces resulting from agitation and aeration schemes in large scale industrial bioreactor. Cell immobilisation has been suggested as a vehicle to protect cells for sustained viability and enhanced productivity. Animal cells have a natural tendency to aggregate and so the immobilisation of cells in microcapsules to provide optimal shear-stress free conditions is considered a promising technique.

Objectives: In the quest to produce a high density CHO DP-12 cell culture, the CHO DP-12 cell line will be cultured in alginate-poly-L-lysine-alginate (APA) microcapsules under batch cultivation conditions. All cellular activities will be compared to those observed when the cells were cultured in suspension. It is hypothesised that enhanced growth and productivity may be achieved due to protection from shear stresses the capsule environment provides. Growth may also be promoted by the presence of high concentrations of growth promoting autocrine factors in the immediate vicinity of the immobilisation matrix. The quality (glycosylation and/or aggregation) of the rIgG1 protein harvested from the encapsulated cultures will also be determined. A thorough investigation of the effect of encapsulation as a technique on recombinant protein quality is yet to be completed. Protein glycosylation may be affected by possible different growth and production activities displayed by the cells. Depending on the permeability of the microcapsules to the rIgG1 protein, aggregation of the protein may be affected through the association of high concentrations in the immobilisation matrix. The results of the encapsulation experiments may in turn outline different CPP's required for monitoring and controlling in encapsulated cultures in comparison to suspension cultures.

Aim 4: Design of an appropriate perfusion system for encapsulated cultures: Implications on rIgG1 quality

Background: The complete colonisation of microcapsules with encapsulated cells has a number of limitations, including the prevalence of the immobilisation matrix in the core of the capsule. For liquid core microcapsules, such as the APA microcapsules, space limitation is not an associated issue. It is hypothesised that cell growth and capsule colonisation may also become limited due to nutrient depletion which typically occurs during batch cultivation schemes. The APA microcapsules themselves fulfil the one main requirement of any

effective perfusion system in that they provide a simple and efficient mechanism for cell retention inside the culture vessel and subsequently allow ease of perfusion of fresh and spent media to and from the culture.

Objectives: Based on the results of the batch encapsulated cultures, and also the metabolic knowledge gained in Aim 1, a suitable perfusion system will be designed for such cultures enabling the addition of growth limiting nutrients and the removal of toxic, inhibitory by-products. It is hypothesised that further enhancements in cell and product yields may be achieved than those which were observed in the batch encapsulated cultures. Any implications such a cultivation scheme may have on the quality (glycosylation and/or aggregation) of the rIgG1 will be monitored.

Chapter 2: Materials and Methods

This chapter reviews the standard instruments and methods that were used routinely throughout the work.

Abbreviations

AFM	Atomic force microscopy
APS	ammonium peroxodisulfate
CHO	Chinese hamster ovary
DTT	dithiothreitol
GU	glucose unit
HCl	hydrochloric acid
HILIC	hydrophilic interaction liquid chromatography
IgG1	Immunoglobulin G1
L-GLN	L-glutamine
L-GLU	L-glutamate
NaOH	sodium hydroxide
SEC	size exclusion chromatography
TEMED	<i>N,N,N,N'</i> -tetramethyl-ethylenediamine

2.1: Cell line and inoculum development

As a recombinant glycoprotein production model, a Chinese hamster ovary (CHO) DP-12 cell line, producing recombinant immunoglobulin G1 (IgG1) was selected for these studies. These cells were transfected to secrete recombinant IgG1, a glycoprotein of molecular weight 150 kDa. Cells, from a working cell bank stored at -196 °C, were rapidly thawed at 37 °C. The cells were used to inoculate a T-flask (75 ml) containing 10 ml of ExCell 325 PF CHO Serum-Free medium (w/o L-glutamine) (Sigma Aldrich #: 24340 C) supplemented with 4 mM L-glutamine (L-GLN) (Sigma Aldrich #: G7513) and 10 mg/L insulin (Sigma Aldrich #: 91077C) at an initial cell density of 1×10^6 cells/ml. After incubation at 37 °C and 5 % CO₂ for 6 hours, the 10 ml of culture was transferred to a 125 ml Erlenmeyer shake flask. A sufficient quantity of media was added to the 125 ml Erlenmeyer shake flask resulting in an initial cell density of 0.3×10^6 cells/ml. The cells were incubated

in a non-gassed incubator, at 37 °C, 100 rpm agitation in non-vented shake flasks. Cell growth was monitored for 5 days. For each of the 5 days, a sufficient quantity of culture media was added to the flask in order to adjust the cell density to $0.3 * 10^6$ cells/ml. On day 5, the cells were harvested and subculture into 30 ml of ExCell 325 PF CHO Serum-Free medium (w/o L-glutamine) supplemented with 4 mM L-glutamine, 10 mg/L insulin and 1 μ M methotrexate (MTX) (Sigma Aldrich #: M9929) at an initial cell concentration of $0.3 * 10^6$ cells/ml. Upon reaching a cell density of $\sim 2 * 10^6$ cells/ml, the cells were harvested by centrifugation and used to inoculate a 500 ml Erlenmeyer shake flask containing 150 ml of culture medium.

2.2: Cell cultures

2.2.1: Cultivation of CHO DP-12 cells in varying concentrations of L-GLN, L-GLU and Ammonia

Small-scale experiments for cells grown in varying concentrations of L-GLN, L-glutamate (L-GLU) and Ammonia were performed in 1 L Erlenmeyer shake flasks with a working volume of 350 ml, incubated at 37 °C, 0 % CO₂ and 100 rpm agitation. For such experiments exponentially growing cells were centrifuged at 200 g for 5 mins and resuspended in the appropriate fresh medium for each test case in triplicate at a seeding density of $0.3 * 10^6$ cells/ml.

2.2.1.1: Culture medium

Cells were cultured in ExCell CHO 325 PF media supplemented with 10 g/L insulin and 1 μ M MTX and 1:100 Penicillin/Streptomycin (Sigma Aldrich cat #: P0781). Both insulin and MTX were prepared as working stock solutions (Section 2.2.1.2). For L-GLN cultures, the concentration of L-GLN in the media was varied in the ranges of 0 mM, 4 mM and 12 mM. The media was supplemented with L-GLN from a 200 mM stock solution. For L-GLU cultures, media was supplemented with a final concentration of 4 mM L-GLU. A L-GLU stock solution was prepared by adding 2 g of L-Glutamic acid (Sigma Aldrich #: G8415) to 20 ml of 1 M hydrochloric acid (HCl). The pH of the solution was adjusted to 7.2 by adding 15 ml of 2 M sodium hydroxide (NaOH) resulting in a final concentration of 388 mM. 33 ml of ultra pure H₂O was subsequently added to the L-GLU stock solution, resulting in a final concentration of 200 mM. For Ammonia cultures, the concentration of ammonium

chloride in the media was varied in the ranges of 5 mM and 15 mM. The media was also supplemented with 4 mM L-GLN. A 1 M Ammonium chloride stock solution was prepared by adding 1.34 g of Ammonium chloride (Sigma Aldrich #: 9434) to 25 ml of ultra pure H₂O. The pH of the solution was adjusted to 7.2 using 2 M NaOH. The concentration of the stock solution was adjusted to 200 mM and 500 mM using ultra pure water for supplementation of the media with a final concentration of 5 mM and 15 mM Ammonia respectively.

2.2.1.2: Preparation of insulin and methotrexate (MTX) stock solution

A 500X (5 mg/ml) working stock solution of insulin was routinely prepared by adding 10 mg Insulin, Recombinant human 5f to 10 ml of a 0.01 M HCl solution. The solution was sterile syringe filtered (0.22 µm Fisher Scientific cat #: FDM-340-010U). The stock solution was added to culture medium directly before use in order to achieve a concentration of 10 mg/L insulin in media.

A 2.2 mM MTX stock solution was prepared by adding 16.5 mg of MTX to 16.5 ml of 1 M NaOH. The solution was sterile filtered and added to culture medium directly before use in order to achieve a concentration of 1 µM MTX in the media.

2.2.2: Cultivation of CHO DP-12 cells in suspension in bench top minifors reactor under batch cultivation conditions

For experiments at larger scale, cells were grown in 1.7 L (1.2 L working volume) Minifors reactors (Infors, Bottmingen, Switzerland). Cells were cultured in ExCell CHO 325 PF media supplemented with 4 mM L-GLN, 10 g/L insulin and 1 µM MTX, 0.01 % antifoam C (Sigma Aldrich #: 59920C). Cultures were carried out at 37 °C and 100 rpm agitation. The minifors reactor was operated under conditions of 95 % DO by means of sparging with compressed air (flowrate of 0.06 L/min). The pH was also controlled at 7.2 through automatic addition of 2 M NaOH, via a feed line, or CO₂ sparging (through the headspace at a flowrate of 0.047 vvm).

Batch suspension culture experiments were carried out in triplicate in order to identify the growth, metabolism and rIgG1 production kinetics and quality of the CHO DP-12 cell line. For such experiments exponentially growing cells in 500 ml Erlenmeyer shake flasks were centrifuged at 200 g for 5 mins and resuspended in the appropriate fresh medium in order to inoculate the reactor at a seeding density of 0.3×10^6 cells/ml.

No data was found in literature concerning ExCell CHO 325 PF medium density or kinematic viscosity. These values correspond to average values of other culture medium, as utilised for similar maximum shear rate in similar stirred tank configured reactors by Fernandes-Platzgummer et al., (2011).

Table 2.2.2.1: 1.7 L minifors reactor characteristics and culture medium properties

Parameter	Value
Working volume (V_L)	1.2 L
Vessel diameter (D_T)	10.5 cm
Impeller diameter (D_i)	6.7 cm
Impeller thickness (T_i)	0.13 mm
Impeller width (W_i)	6.9 cm
Culture medium density (ρ)	1.005 g/ml
Culture medium kinematic viscosity (ν)	0.0092 cm ² /s

2.2.2.1: Estimation of maximum shear stress under stirred conditions in 1.7 L minifors reactor

The maximum shear stress under stirred conditions in the 1.7 L minifors reactor was determined based on the work presented by Fernandes-Platzgummer et al., (2011), in which the effect of shear stress, brought about by stirred conditions, on a mouse embryonic stem cell line was determined. Theoretical values of maximum shear stress under stirred conditions, τ_{\max} (dyn/cm²), can be determined through the evaluation of Equation (2.2.2.1.1). The equation assumes that the shear stress created by impeller rotation is a result of flow through Kolmogorov eddies. Agitation results in the formation of turbulent eddies which dissipate energy by disintegrating into Kolmogorov eddies. (Cherry and Kwon, 1990; Yoon et al., 2005; Abranches et al., 2006).

$$\tau_{\max} = 5.33 \cdot \rho \cdot (\varepsilon_i \cdot \nu)^{1/2} \quad (2.2.2.1.1)$$

where ρ denotes fluid density and ν denotes the kinematic viscosity. ε_t is the total energy dissipated per unit mass and in sparged conditions is defined by equation (2.2.2.1.2) as described by Fernandes-Platzgummer et al., (2014).

$$\varepsilon_t = \varepsilon_{is} + \varepsilon_s \quad (2.2.2.1.2)$$

ε_s is the energy dissipation rate from the sparging given by:

$$\varepsilon_s = v_s \cdot g \quad (2.2.2.1.3)$$

where v_s denotes the superficial gas velocity (cm/s) and g is the gravitational constant (981 cm/s²). v_s is determined by the following equation:

$$v_s = \frac{4Q_{air}}{\pi D_i^2} \quad (2.2.2.1.4)$$

where Q_{air} is the air flow rate (cm³/min) and D_i in the impeller diameter.

ε_{is} is the energy dissipation rate from the impeller under sparged conditions given by:

$$\varepsilon_{is} = \frac{P_s}{V_L \rho} \quad (2.2.2.1.5)$$

where V_L is the working volume of the reactor and ρ is the fluid density. P_s is the power consumption under sparged conditions and may be determined from the following equation:

$$P_s = Np_s N^3 D_i^5 \rho \quad (2.2.2.1.6)$$

where N is the agitation rate (s⁻¹). Np_s is the dimensionless power number under sparged conditions. As described by Fernandes-Platzgummer et al., (2014), $Np_s \approx Np$ for air flowrates typically used in mammalian cell culture. The power number Np can be calculated from Nagata correlations (Sen et al., 2002), as described by Fernandes-Platzgummer et al., (2011).

$$Np = \frac{K_1}{Re} + K_2 \cdot \left[\frac{(10^3 + 1.2 \cdot Re^{0.66})}{10^3 + 3.2 \cdot Re^{0.66}} \right]^{K_4} \quad (2.2.2.1.7)$$

where,

$$K_1 = 14 + \left(\frac{W_i}{D_t}\right) \left[670 \cdot \left(\frac{D_i}{D_t} - 0.6\right)^2 + 185 \right] \quad (2.2.2.1.8)$$

where,

$$K_2 = 10^{K_3} \quad (2.2.2.1.9)$$

$$K_3 = 1.3 - 4 \cdot \left(\frac{W_i}{D_t} - 0.5\right)^2 - 1.14 \cdot \left(\frac{D_i}{D_t}\right) \quad (2.2.2.1.10)$$

$$K_4 = 1.1 + 4 \cdot \frac{W_i}{D_t} - 2.5 \cdot \left(\frac{D_i}{D_t} - 0.5\right)^2 - 7 \cdot \left(\frac{W_i}{D_t}\right)^4 \quad (2.2.2.1.11)$$

where W_i is the impeller width, D_t is the vessel diameter and Re is the Reynolds number.

$$Re = \frac{D_i^2 \cdot N}{\nu} \quad (2.2.2.1.12)$$

2.2.3: Cultivation of CHO DP-12 cells in Alginate-poly-L-lysine-alginate microcapsules in bench top minifors reactor and 1 L Erlenmeyer shake flasks under batch and control-fed perfusion cultivation conditions

2.2.3.1: Alginate-poly-L-lysine-alginate microcapsule formation

Microcapsules were prepared under completely sterile conditions using an encapsulation device (Encapsulator® Inotech, Dottikon, Switzerland). This instrument may be described as a droplet generator which utilises laminar jet break up technology for the production of highly monodisperse small droplets (200 µm to 1500 µm). The device is comprised of a 2-litre glass reaction vessel with stainless steel top and bottom plates and attached agitator. The top plate contains a nozzle attached to a vibrating device. The vibrating device is insulated from the surrounding structures by rubber mounts in order to avoid the generation of resonance frequencies in the system. A feed line connects the nozzle to the

polymer solution vessel. The flow of alginate from the polymer vessel to the nozzle is achieved through the delivery of compressed air to the headspace of the polymer vessel to provide a headspace pressure of 0.1-2 bar. By regulating the pressure it is therefore possible to control the flow rate of alginate through the nozzle, allowing the extrusion of large volumes of polymer (1-2 L/h).

The main steps of the microcapsule production procedure such as precipitation, gellation, membrane coating and washing of the microcapsules are performed in the glass reaction vessel. The base plate of the vessel houses a porous steel disc, connected to an outlet enabling the withdrawal of the different solutions used in the step-wise production of the microcapsules, while retaining the capsules inside the vessel. A second port on the top plate is connected to a feed line which enables the connection of vessels containing buffers required in microcapsules formation to be attached to the glass vessel. The flow of buffers to the glass vessel is also achieved through the delivery of compressed air to the headspace of the buffer vessel. The glass vessel, feed lines and polymer and buffer vessels are all fully autoclavable, enabling the complete microcapsule production process to be performed under sterile conditions.

For the production of microcapsules, the alginate is supplied from the polymer vessel to the nozzle at a set feed rate in order to obtain a jet of liquid. A magnetically operated cup is suspended from the top plate and may be positioned directly below the nozzle. The cup enables the retrieval of any initial polymer drops during priming of the nozzle. A sinusoidal vibration frequency is applied to the 300-400 μm diameter nozzle, which disrupts the flow of alginate, achieving jet break-up into droplets. An electrical potential is then applied between the nozzle and stainless steel ring placed below (~30 mm) the nozzle outlet. The ring is electrically insulated from the nozzle and reactor vessel. Due to the electrical charge applied between the steel ring and the nozzle, the alginate drops have a highly negatively charged surface. As they pass through the steel ring, they are deflected away from the vertical and so impact with the gelling solution over a wider defined area. The result of the applied electrical potential is an improvement in the formation and monodisperse of the regular droplets. The formation of the monodispersed droplets can be observed through the glass vessel with the aid of a stroboscopic light, the frequency of which is directly related to the one applied to the nozzle. Vertical dropping beads would impact the gelling solution in the same area and thus impact each other resulting in the formation of nonspherical, coalescent drops (Serp et al., 2000).

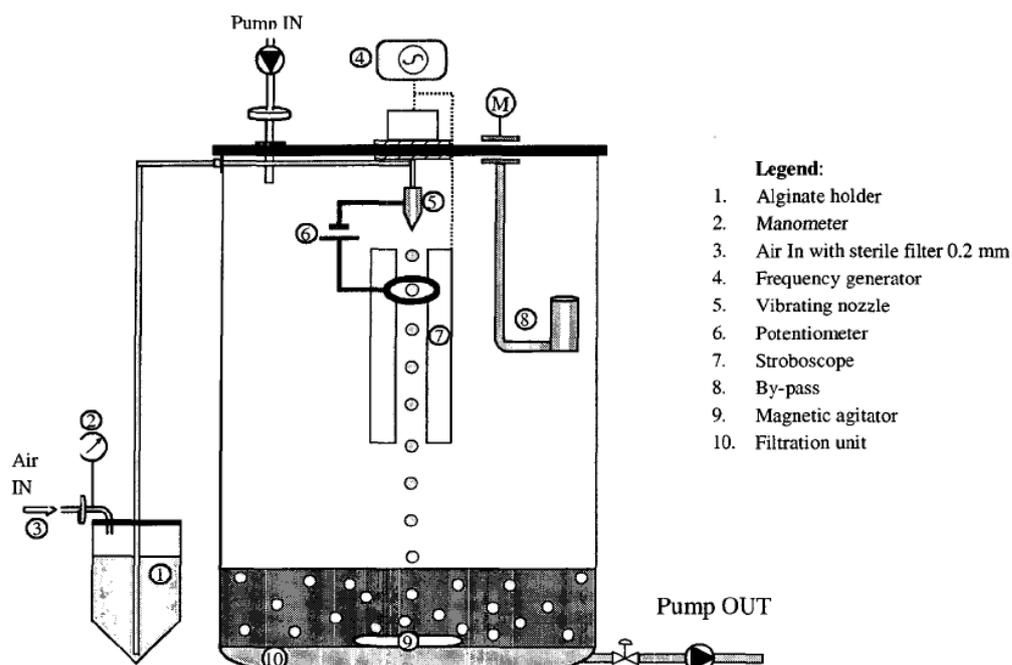


Figure 2.2.3.1: Schematic representation of the Inotech Encapsulator® according to (Gugerli, 2003).

2.2.3.2: CHO DP-12 cell encapsulation in Alginate-poly-L-lysine-alginate microcapsules

All encapsulation experiments carried out in the 1.7 L minifors reactor required a total volume of 300 ml of alginate containing CHO cells per run. Due to the time taken to extrude 300 ml of alginate, the process was completed in 2 batches of 150 ml alginate containing CHO cells. All encapsulation experiments carried out in 1 L Erlenmeyer shake flasks required a total volume of 100 ml of alginate containing CHO cells per flask. All of the microcapsules required to seed 1 flask could be prepared as one batch.

Exponentially growing cells in suspension were centrifuged at 200 g for 5 minutes and resuspended in the required volume of pre-sterilized (0.2 µm microfiltration) sodium alginate solution (1.5 % w/v, Keltone LV Inotech Switzerland) at a density of 0.5×10^6 cells/ml sodium alginate. The required volume of alginate was dependent upon the experiment which the microcapsules would be used in. For minifors reactor experiments the required volume of alginate was 300 ml to be extruded in 150 ml batches (X2). For 1 L Erlenmeyer shake flask experiments the required volume was 100 ml per flask, which could be extruded all at once. Calcium Alginate (Ca-alginate) beads were formed by extrusion of the sodium alginate solution, containing the CHO cells, through the encapsulator nozzle (diameter 300 µm) into the encapsulator glass vessel containing 1L of a 110 mM aqueous CaCl₂ solution (pH 7.0)

(Sigma Aldrich #: C5670). The reaction was stopped after 10 minutes by the withdrawal of the CaCl_2 solution through the porous steel disc of the reaction vessel. The beads were washed in copious amounts of a saline buffer (0.9 % NaCl, 10 mM MOPS (Sigma Aldrich #: M3183), pH 7.0) in order to remove any un-reacted alginate material. For 1.7 L minifors reactor experiments this first batch of beads extruded (150 ml) was transferred to a second reaction vessel in the saline solution by means of a feed line connected to a second port at the base of the first reaction vessel. The second batch of 150 ml beads were then prepared as above in the first reaction vessel and subsequently transferred in saline solution to the second reaction vessel. All of the following steps for the preparation of capsules were performed on the 300 ml of alginate beads containing CHO cells in the second reaction vessel. For 1 L Erlenmeyer shake flask, as the total volume of alginate for the experiment could be extruded in one batch, and so all of the subsequent microcapsule preparation steps could be performed in the first reaction vessel. There was no need for a second reaction vessel for these experiments. Capsules were formed by the addition of 1.2 L per 300 ml beads prepared of a 0.05 % poly-L-lysine (PLL) (Sigma Aldrich #: P2636) in 110 mM CaCl_2 solution. The PLL was labelled with a fluorescent dextran to enable visualisation of the membrane (Sigma Aldrich #: F7250) The molecular weight of the PLL used was 30,000-75,000 kDa. After incubation at room temperature for 30 minutes with gentle mixing, the beads were again washed with saline buffer, followed by incubation in 1 L, per 300 ml beads prepared, of 0.03 % (w/v) sodium alginate for 10 minutes. The solid alginate beads formed were again washed with saline buffer. Liquefaction of the solid alginate beads to form capsules was achieved through incubation of the capsules in 0.05 M sodium citrate (Sigma Aldrich #: S1804) for 15 minutes with gentle mixing. The resulting microcapsules were washed once with saline buffer and equilibrated once with ExCell CHO 325 PF media. Finally the microcapsules were immersed in an equal volume of ExCell CHO 325 PF media, supplemented with 4 mM L-GLN, 10 mg/L insulin and 1 μM MTX. The microcapsules and media were transferred into an inoculation bottle attached to the fully porous port in the base plate of the first or second reaction vessel, depending on whether all of the microcapsule preparation was being conducted in the first or second reaction vessel. The microcapsules were immediately used to inoculate the 1.7 L Minifors reactor or 1 L Erlenmeyer shake flasks. All batch encapsulated culture experiments were also carried out in triplicate. The control-fed perfusion experiment carried out in the 1.7 L minifors reactor was carried out only once. Control-fed perfusion experiments carried out in 1 L Erlenmeyer shake flasks were carried out in

triplicate. A capsule volume of 300 ml per 900 ml of culture medium (ratio 1:3) was used for the 1.7 L minifors reactor cultures. A ratio of 100 ml of capsules per 300 ml of culture media was used for 1 L Erlenmeyer shake flask cultures.

2.2.3.3 Bioreactor and shake flask conditions for encapsulated cultures

All 1.7 L minifors reactor conditions were maintained as explained above, under Section 2.2.3.1, for all batch and the control-fed perfusion cultures carried out. The batch and control-fed perfusion cultures performed in 1 L Erlenmeyer shake flask cultures were incubated under conditions of 37 °C, 100 rpm agitation in a non-CO₂ incubator. Such cultures were performed in non-vented 1 L Erlenmeyer shake flasks. A control-fed perfusion culture in 1 L Erlenmeyer shake flasks was also carried out in triplicate in a 5 % CO₂ incubator under conditions of 37 °C and 100 rpm agitation.

2.2.3.4: Control-fed perfusion strategy

All control-fed perfusion cultures acted as batch cultures essentially until day 5 of the culture period. Perfusion was accomplished by removing one quarter of the volume of the total culture volume in microcapsule free spent media and replacing it with the same volume of fresh media. Fresh media was ExCell CHO 325 PF supplemented with 10 g/L insulin and 1 µM MTX. In 1.7 L minifors reactor cultures, the agitation was switched off in the bioreactor to allow the microcapsules to settle. The spent media was subsequently removed via a harvest line connected to a graduated Duran bottle. The harvest line was positioned at a level in the minifors reactor so as that it was immersed in media only and did not make contact with the settled microcapsule bed formed at the bottom of the reactor. Immediately after fresh media was added, the agitation in the reactor was re-started. It was estimated that the entire perfusion procedure took no longer than 5 minutes. Perfusion was performed once daily for the control-fed perfusion culture carried out in the 1.7 L minifors reactor culture.

In 1 L Erlenmeyer shake flask cultures, shake flasks were removed from their relevant incubators and aseptically placed in a Biosafety cabinet. After approximately 1 minute, 100 ml of spent media was removed from the shake flask aseptically using a graduated pipette. The media was removed by allowing contact of the pipette with the surface of the media present in the flask. Care was taken not to remove capsules which had settled at the bottom of the shake flask. The same volume of fresh media was then added to each flask using aseptic technique. Fresh media was ExCell CHO 325 PF supplemented with 10 g/L insulin

and 1 μM MTX. Flasks were immediately returned to their respective incubators. Perfusion was performed once daily for unvented shake flask cultures incubated in the non- CO_2 incubator and twice daily from day 9 onwards for the vented shake flask cultures incubated in the CO_2 incubator.

Prior to perfusion, sampling of the cultures was performed, Section 2.3 below. Sampling enabled enumeration of cell numbers as well as glucose, glutamine and ammonia concentrations to be determined. Based on cellular growth rates and specific metabolite consumption and production rates calculated, the concentration of glucose and glutamine in the fresh medium was adjusted. The adjustments were made to meet the concentrations that would be required by a forecasted predicted cell number which would be present in the culture on the following day based on the growth rate displayed by the cells. (See Section 2.9 for calculations). Additional glucose was added by dissolving glucose anhydrous (Sigma Aldrich #: 158968) in 7 ml of cell culture grade water. The solution was sterile filtered (0.22 μm) prior to addition to the pre-sterile filtered fresh perfusion medium. The volume of glucose solution being added to the fresh medium was kept constant each day in order to keep the dilution factor of the medium constant. The amount of glucose added to the water was adjusted according to the cells requirements. The desired concentration of L-glutamine in the fresh media was added from a 200 mM stock solution. Once stationary growth had been established the glucose and glutamine concentrations were added to meet the requirements of the non-growing cells based on the calculated specific consumption rates during this culture period.

The perfusion rate was only adjusted if the ammonia concentration in the control-fed perfusion culture approached > 5 mM (i.e. the maximum concentration usually present in a batch culture).

2.3. Samples analysis

Sampling of the shake flask cultures and Minifors reactor cultures was completed on a daily basis for biomass concentration determination, concentration of culture metabolites, IgG1 purification and quantitation. Biomass concentration was determined in a daily basis. Culture supernatants (4 ml) were aliquoted after centrifugation and filtration (0.45 μm) and stored frozen at -80 °C for later analysis. From day 4 onwards, ~ 10 ml of culture supernatant was stored at -80 °C after centrifugation for purification of IgG1 at a later stage. Sampling of encapsulated cultures also involved capsule size and capsules strength determination.

2.3.1: Biomass determination in suspension cultures

Cell concentration and viability was determined microscopically using the Trypan blue exclusion method. Cultures samples were initially diluted 1: 2 with 0.4 % trypan blue solution (Sigma Aldrich #: T8154). The dilution factor was increased with increasing biomass concentrations. The total and viable cell numbers were counted microscopically using a Neubauer haemocytometer.

2.3.2: Biomass determination in encapsulated cultures

Extracapsular biomass was determined microscopically using the trypan blue exclusion method according to 2.3.1. Intracapsular biomass concentration determination firstly involved release of the cells from the microcapsules. Microcapsules were separated from culture media in offline samples using a cell sorter. The cell sorter device consisted of a mesh which was impermeable to particles >100 μm , therefore ideal for separating microcapsules from culture media. The separated capsules were washed with 1X PBS to remove any extracapsular cells present on the surface of the microcapsules. A known volume of the microcapsules, mixed with a known volume of 1X PBS in a 1 ml syringe, was then extruded through a 300 μm needle 3 times, in order to dissociate cell clusters if present. Biomass concentration was subsequently determined using the trypan blue exclusion method outlined in 2.3.1.

2.3.3: Metabolite determination

Glucose and lactate concentrations were determined by HPLC analysis using a Supelco H column, H_2SO_4 as solvent and a RI detector for all batch cultures. As the controlled perfusion cultures required immediate quantification of glucose present in spent media samples, the glucose concentration present in the media was determined using a commercially available enzymatic kit (Sigma Aldrich #: GAGO20-1KT). Glucose present in samples is oxidised to gluconic acid and hydrogen peroxide. O-dianisidine subsequently becomes oxidised in a reaction with the hydrogen peroxide and forms a coloured compound (brown). A more stable coloured product (pink) is formed upon addition of sulphuric acid to the samples. The intensity of the pink colour measured at 540 nm is proportional to the

concentration of glucose present in the sample. Glucose concentrations in samples are determined from a standard curve prepared from glucose solution provided in the kit.

Ammonia and L-GLN concentrations were determined enzymatically using a commercially available kit (Megazyme; L-Glutamine/Ammonia (Rapid) K-GLNAM 10/08). L-glutamine present in samples is deaminated in the presence of Glutaminase to L-glutamate and ammonium ions. In the presence of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and glutamate dehydrogenase (GIDH), the ammonia formed in the initial reaction, reacts with 2-oxoglutarate to form L-glutamate and NADP^+ . The amount of NADP^+ formed is stoichiometric with the amount of glutamine and ammonia. NADPH consumption is measured by the decrease in absorbance at 340 nm.

2.3.4: Capsule size determination

Capsule size determination was carried out using a laboratory microscope (Nikon Eclipse TI) with the corresponding software for image analysis (NIS-element AR). Thirty capsules were measured individually to calculate the average radius and standard deviation, as estimated by comparison with a calibrated scale. Generally size distribution was below 3 % between microcapsules in a given culture conditions at a given time.

2.3.5: Mechanical strength of microcapsules using a Texture analyser

Capsule mechanical resistance was routinely measured throughout each of the encapsulated culture runs by means of a Texture analyzer (TA.XT plus, Stable Micro Systems, United Kingdom). The instrument consists of a mobile piston driven at a constant speed. A layer of capsule beneath the piston may be compressed until deformation of the capsules is achieved. The force required by the piston to deform the capsules is representative of the capsules mechanical resistance. The mechanical resistance is represented as Force (g)/capsule. The average number of capsules in the layer compressed is determined using the following equation, assuming a hexagonal disposition of the capsules under the probe:

$$n_{caps} = \frac{S_{probe}}{2 \cdot \sqrt{3} \cdot r_{caps}^2} \quad (2.3.5.1)$$

With n_{caps} being the number of capsules under the piston at deformation ε , S_{probe} is the probe surface and r_{caps} is the radius of the capsules at deformation. The average force on each capsule is determined by dividing the total measured strength by the number of capsules.

The determination of the capsule radius at deformation, r_{caps} , is based on the deformation model described by Gugerli, (2003).

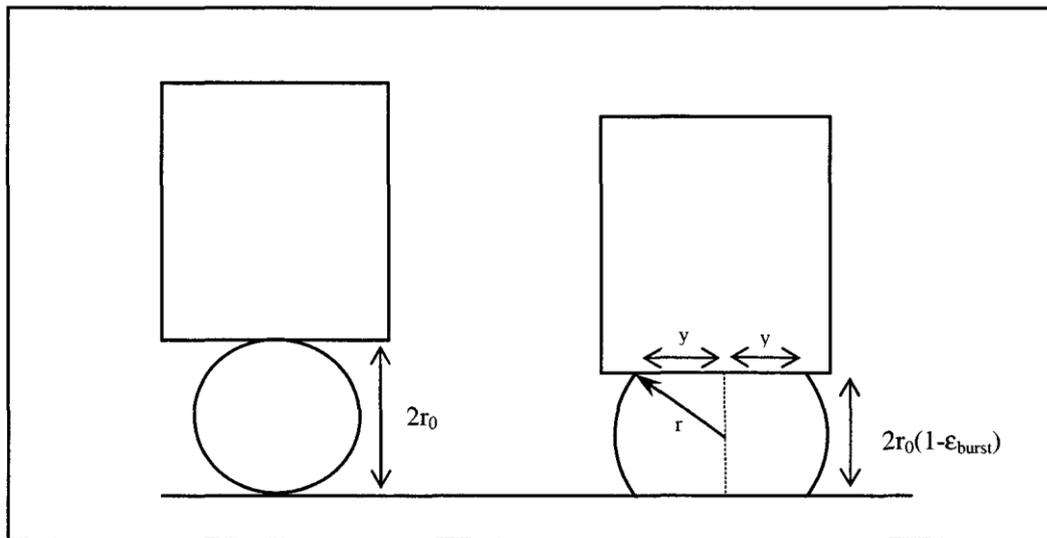


Figure 2.3.5.1: Schematic compression of a capsule by Texture Analyser piston (Gugerli, 2003).

When a capsule is under compression, the radius may be determined by Pythagoras theorem in which, $r_{compression}$, is the hypotenuse of a right angled triangle formed in Figure 2.3.5.1 above. The opposite is $r_0(1 - \varepsilon_{burst})$ and adjacent is given by y .

By Pythagoras theorem:

$$(r_{compression})^2 = (y)^2 + (r_0(1 - \varepsilon_{burst}))^2 \quad (2.3.5.2)$$

$$\varepsilon = \frac{l}{l_0} \quad (2.3.5.3)$$

With l_0 being the initial diameter of the microcapsules and l being the compression distance. l is determined by calculating the difference in distance travelled by the piston between

when the piston comes into contact with the capsules and that observed when the capsules are at 100 % deformation.

y may subsequently be determined by the following equation for the volume of a capsules:

$$V = \frac{4}{3}\pi r_0^3 = y^2 \pi x + y \left(\frac{\pi x^2}{4} \right) + \left(\frac{\pi x^3}{6} \right) \quad \text{with } x = 2r_0(1 - \varepsilon_{burst}) \quad (2.3.5.4)$$

Once the number of capsules has been enumerated, Equation 2.3.6.1, the average force imposed on each capsule may be determined by dividing the total measured strength by the number of capsules.

2.3.6: Determination of capsule permeability to rIgG1 protein

Capsules removed from cultures were washed in PBS (1X). 300 μ l of capsules were immersed in 600 μ l of a dextran standard solution (ranging in molecular weight from 10 kDa-500 kDa; Sigma Aldrich #: FD10S; FD70S; FD150S; FD250S; FD500S). Dextran standard solutions were prepared at a concentration of 0.05 % w/v in PBS. Immediately after addition of dextran standards to the microcapsules, 300 μ l of the liquid was withdrawn and filtered (0.45 μ m) for analysis at 450 nm. The concentration of dextran in the solution could be enumerated from a standard curve prepared for each molecular weight dextran analysed. This value served as the initial concentration denoted by C_0 . The capsules immersed in dextran standard solution were incubated at room temperature under agitation for 2 hours. Following the incubation period, the supernatant was removed from the capsules and filtered (0.45 μ m) for analysis at 450 nm. Concentrations were enumerated using the standard curve. The value for the concentration of dextran in the solution after incubation was denoted by C_f . The ratio of $\frac{C_0}{C_f}$ was determined. Values equal to 1 are indicative of no diffusion of

dextran into microcapsules as the concentration of dextran in the solution surrounding the sample is the same before and after incubation. Values approaching 1.5 are indicative of free diffusion, Values between 1.5 and 1 indicate permitted diffusion of the dextran, however with difficulty. Confirmation of movement of the dextran from the surrounding liquid, into the capsules was made using confocal microscopy analysis.

2.3.7: IgG1 Purification and quantification

rIgG1 was purified from culture supernatants using commercially available high affinity Protein A/G Nab spin column kits (Thermo Scientific PN89948). The CHO supernatant was thawed and purified immediately as per manufacturer's instructions. Briefly, the Protein A/G Nab spin columns are equilibrated with Binding Buffer (100 mM phosphate, 150 mM sodium chloride) (2x400 μ l). The pre-equilibrated columns are loaded with 500 μ l of CHO supernatant and incubated at room temperature for 10 minutes with end-over-end mixing. The spin column is placed in a collection tube and centrifuged for 1 minute to remove the non bound sample components. The column is subsequently washed with Binding Buffer (3x400 μ l). The rIgG is eluted from the column into a new collection tube with IgG Elution Buffer (3x400 μ l). The elution buffer is neutralised with Neutralisation Buffer (1 M Tris•HCl) (40 μ l) Eluted rIgG1 was quantified immediately using nanodrop A 280 nm. The purified protein samples were stored at -80 °C for glycan analysis at a later stage.

The concentration of protein in purified samples was later verified using SEC analysis using an Aquity UPLC BEH200 1.7 μ m 4.6x150 mm column. A mobile phase containing 50 mM sodium phosphate buffer at pH 8.0 was used to identify the IgG1 peak over a run time of 6 minutes. The flow rate was 0.4 ml/min and the column was maintained at ambient temperature. Protein was detected by UV 280nm. A standard curve of IgG1 (Sigma Aldrich #: 14506) was prepared by making dilutions of the 10 mg/ml IgG1 standard in water resulting in a range of concentrations from 0.5 mg/ml to 5 mg/ml. The standard curve was prepared by plotting the peak area detected vs. the protein concentration in the standard samples. The equation generated from this plot was used to enumerate the concentration of IgG1 in the purified culture samples. The average error between the concentrations of IgG1 detected using the nanodrop and SEC ranged from 0.05 % – 9 %, with a trend for decreasing error as the concentration of purified protein in the sample increased.

2.3.8: IgG1 N-glycan analysis

The N-glycan profiles of the samples were characterized using hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection

2.3.8.1: Glycan release and labelling

IgG samples (100 μ g) were reduced in a polypropylene 96-well flat-bottomed microplate by adding 2 μ l of 5 \times sample buffer (0.625 ml of 0.5 M Tris [pH 6.6], 1 ml of 10%

SDS, and 3.375 ml of water), 2 μ l of water, and 1 μ l of 0.5 M dithiothreitol (DTT) and subsequently incubated at 65 °C for 15 min. The samples were then alkylated by adding 1 μ l of 100 mM iodoacetamide and were incubated for 30 min in the dark at room temperature. The sample was then set into a gel block by adding 22.5 μ l of 30% (w/w) acrylamide/0.8% (w/v) bis-acrylamide stock solution (37.5:1.0, Protogel, National Diagnostics, Hessle, Hull, UK), 11.25 μ l of 1.5 M Tris (pH 8.8), 1 μ l of 10% SDS, 1 μ l of 10% ammonium peroxydisulfate (APS), and finally 1 μ l of *N,N,N,N'*-tetramethyl-ethylenediamine (TEMED), mixed, and then left to polymerize. The gel blocks were transferred to a filter plate (Whatman protein precipitation plate) and then washed with 1 ml of acetonitrile with vortexing on a plate mixer (Sarstedt, Leicester, UK) for 10 min, followed by removal of the liquid (all washes and elutions were performed on a vacuum manifold). This washing procedure was repeated twice with 1 ml of 20 mM NaHCO₃ (pH 7.2) followed by 1 ml of acetonitrile. The gel pieces were dried in a vacuum centrifuge. N-glycans were released by adding 50 μ l of 0.1 U/ml PNGaseF (Prozyme, Leandro, CA, USA) in 20 mM NaHCO₃ (pH 7.2) to reswell the gel pieces for 5 min, 50 μ l of 20 mM NaHCO₃ (pH 7.2) was added, and then the plates were sealed with adhesive film (SealPlate, Web Scientific, Crewe, UK) and incubated overnight at 37 °C. The released glycans were collected in a 2-ml square tapered polypropylene 96-well plate by washing the gel pieces with 3 \times 200 μ l of water, 200 μ l of acetonitrile, 200 μ l of water, and finally 200 μ l of acetonitrile. The released glycans were dried, 20 μ l of 1% formic acid was added, and the mixture was incubated at room temperature for 40 min and then re-concentrated. Samples for HILIC analysis were labeled by adding 5 μ l of 2AB labelling solution (LudgerTag 2AB labelling kit, Ludger, Abingdon, UK), vortexed for 10 min, incubated for 30 min at 65 °C, vortexed again for 10 min, and incubated for a further 90 min. Excess 2AB was removed using Phynexus normal phase chromatographic tips. Duplicate releases were performed for all samples.

2.3.8.2: HILIC Fluorescence

HILIC was carried out on a 3 μ m TSKgel amide-80 (150 mm x 4.6 mm) column as detailed in Royle et al., (2008) with retention times expressed as glucose units (GU). The principle of this method involves the interaction of the charged silanol groups on the amide column with the hydroxyl groups on the carbohydrates in a gradient decreasing acetonitrile. The glycans are eluted based on the hydrophilic surface area exposed to resin. The retention time of the glycan peaks can be compared with an external 2-AB labelled dextran standard

for structural elucidation by converting the retention time of the peaks into GU. The dextran standard is a glucose homopolymer obtained by controlled acid hydrolysis of dextran resulting in homopolymers between 1 – 22 glucose residues. The GU value of the glycan peak is directly related to the number and linkage of its component monosaccharide units.

2.3.8.3: Assignment of GU values

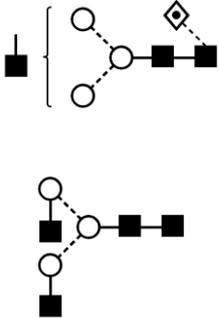
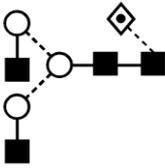
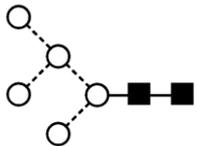
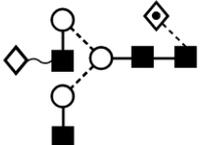
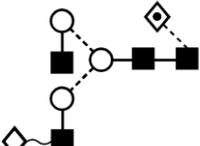
HILIC analysis of the 2AB labelled N-glycan pool released from IgG1 samples purified from cultures separated into a total of 7 main peaks, each with a corresponding GU value as determined by comparison with the dextran standard. The corresponding number and linkage of the component monosaccharide units represented by each GU value was elucidated by carrying out a series of exoglycosidase digestions on one of the released glycans. Each exoglycosidase enzyme has a different specificity resulting in the loss of a distinct monosaccharide from the previously released glycans. The following exoglycosidase enzymes were used:

Digestion Enzyme	Specificity
ABS	Releases $\alpha(2-3)$, $\alpha(2-6)$ and $\alpha(2-8)$ linked non-reducing terminal sialic acid
BTG	Hydrolyses non-reducing terminal $\beta(1-3)$ and $\beta(1-4)$ linked galactose
BKF	Releases $\alpha(1-2)$ and $\alpha(1-6)$ linked non-reducing terminal fucose. Used for release of core $\alpha(1-6)$ fucose residues.
JBH	Releases non-reducing terminal $\beta(1-2,3,4,6)$ linked N-acetylglucosamine and N-acetylgalactosamine residues

HILIC analysis of the exoglycosidase treated glycans results in the loss of specific peaks from the chromatogram which represent a glycan structure containing the monosaccharide which the enzyme used was specific for. The release of a monosaccharide from the glycan confers a new structure to the glycan, resulting in a decrease in the GU value representing the structure which the treated glycan has now conformed to. As a result of exoglycosidase

treatment of the released glycans and use of GlycoBase, the following peaks were identified from the GU values obtained:

Table 2.3.8.3.1: Assignment of corresponding number and linkage of the component monosaccharide units represented by each GU value elucidated by carrying out a series of exoglycosidase digestions on one of the released glycans.

Peak	Structure	UOXF Symbol	GU
1	F(6)A1/A2		5.39 +/- 0.05
2	F(6)A2		5.85 +/- 0.05
3	M5		6.18 +/- 0.05
4	F(6)A2[6]G1		6.64 +/- 0.05
5	F(6)A2[3]G1		6.77 +/- 0.05

6	F(6)A2G2		7.54 +/- 0.05
7	F(6)A2G2S1		8.28 +/- 0.20

2.3.8.4: Glycan nomenclature

All N-glycans have two core GlcNAcs: F at the start of the abbreviation indicates a core $\alpha(1-6)$ fucose linked to the inner GlcNAc; Mx number (x) of mannose on core GlcNAcs; Ax, number of antenna (glcNAc) on trimannosyl core; A2, biantennary with both GlcNAcs as $\beta(1-2)$ linked; A3 triantennary with a GlcNAc linked $\beta(1-2)$ to both mannose and a third GlcNAc linked $\beta(1-4)$ to the $\alpha(1-3)$ linked mannose; A3', triantennary with a GlcNAc linked $\beta(1-2)$ to both mannose and the third GlcNAc linked $\beta(1-6)$ to the $\alpha(1-6)$ linked mannose; A4, GlcNAcs linked as A3 with additional GlcNAc $\beta(1-6)$ linked to a $\alpha(1-6)$ mannose; B, bisecting GlcNAc linked $\beta(1-4)$ to $\beta(1-3)$ mannose; Gx number (x) of $\beta(1-4)$ linked galactose on the antenna; Fx, number (x) of linked fucose on antenna, (4) or (3) after the F indicates that the Fuc is $\alpha(1-4)$ or $\alpha(1-3)$ linked to the GlcNAc; Sx, number (x) of sialic acids linked to galactose; the number 3 or 6 in parentheses after S indicates whether the sialic acid is in an $\alpha(2-3)$ or $\alpha(2-6)$ linkage.

Symbol	Saccharide	Abbreviation	Linkage Type
■	<i>N</i> -acetylglucosamine	GlcNAc	--- known α -linkage
□	Glucose	Glc	— known β -linkage
◇	Galactose	Gal	~·~ unknown α -linkage
◆	<i>N</i> -acetylgalactosamine	GalNAc	~ unknown β -linkage
◇	Fucose	Fuc	
○	Mannose	Man	
★	<i>N</i> -acetylneuraminic acid	NeuNAc (NANA)	
☆	<i>N</i> -glycolylneuraminic acid	NeuNGc (NGNA)	
△	Xylose	Xyl	

Linkage Position

2.3.9: IgG1 aggregation analysis

Approximately 10 μg of purified rIgG1, at a concentration of 1 $\mu\text{g}/\mu\text{l}$, was prepared for aggregation analysis using size exclusion chromatography (SEC). The IgG1 aggregates and monomers were separated by SEC using an Aquity UPLC BEH200 1.7 μm 4.6x150 mm column. The separation was based on an isocratic gradient using 50mM sodium phosphate buffer at pH 8.0 over 6 minutes. The flow rate was 0.4 ml/min and the column was maintained at ambient temperature. Protein was detected by UV 280nm. The peaks were quantified by calculating the relative percentage areas of multimeric, dimeric and monomeric species. The results were subsequently expressed in terms of the relative % abundance of monomeric rIgG1 present in the samples.

2.4: Calculations

The specific growth rate, μ , was determined by plotting the logarithm of the viable cell density $\times 10^6$ (cells/ml) over the cultivation time period (h) during the exponential phase of growth. The specific rIgG1 productivity, q_{rIgG1} , was based on data obtained at the end of the stationary phase of growth and evaluated using the following equation:

$$q_{rIgG1} = \frac{C_{protein(t)} - C_{protein(t_0)}}{X_t - X_{t_0}} \cdot t \quad (2.4.1)$$

With c = quantity rIgG1 present (μg), q = specific production rate ($\mu\text{g}/10^6$ cells/h), X_{t_0} = initial cell number present (10^6 cells), X_t = cell number present at end of stationary phase, i.e. maximum viable cells present (10^6 cells), t = elapsed time (h).

The specific rates of glucose, glutamine, glutamate, lactate and ammonia consumption and production were determined based on data obtained during the exponential growth phase and evaluated using the following equation

$$q_{metabolite} = \frac{C_{metabolite(t)} - C_{metabolite(t_0)}}{X_t - X_{t_0}} \cdot t \quad (2.4.2)$$

With c = quantity of metabolite present (moles), q = specific consumption/production rate of the metabolite, X_{t_0} = initial number of cells present (10^6 cells), X_t = number of cells present (10^6 cells), t = elapsed time (h).

The predicted number of cells which would be present in the bioreactor on the following day was determined for control-fed perfusion cultures using the following:

E.g. On day 5 of a culture period, it can be predicted that the number of cells which would be present in the bioreactor on day 6 were determined using the following equation:

$$\ln(x) = \mu_{\max} \cdot t + \ln(x_0) \quad (2.4.3)$$

With x being the number of cells present in the reactor on day 6, x_0 the number of cells present in the reactor on day 5, μ_{\max} the maximum specific growth rate being displayed by the cells, t the length of time between x and x_0 , (days).

The specific consumption rate being displayed by the cells for glucose and glutamine is enumerated using equation 2.9.2 above. The amount of the nutrient required by the predicted number of cells present on day 6 was calculated using the following equation:

$$Quantity_{metabolite} = q_{metabolite} * x * t \quad (2.4.4)$$

With $q_{metabolite}$ represented the calculated specific consumption rate being displayed by the cells for a given nutrient, x being the number of cells predicted to be present on day 6, t being the length of time between day 5 and 6 (days).

The % colonisation of microcapsules with cells was calculated based on the total number of cells present in the culture expressed as a % of the total theoretical maximum number of cells possible in the volume of capsules in the culture.

The maximum number of cells possible in a capsule may be determined by dividing the volume of a capsule by the volume of a cell using the following equation:

$$\frac{Volume_{capsule}(L)}{Volume_{cell}(L)} \times Kepler's\ Conjective \quad (2.4.5)$$

With the volumes determined using $\frac{4}{3}\pi r^3$, in which r is the radius of the cell or capsule.

The number of capsules present in a given volume of capsules may be calculated using the following equation:

$$\frac{Volume_{alginate}}{Volume_{capsule}} \times Kepler's\ Conjective \quad (2.4.6)$$

Therefore the total theoretical maximum number of cells possible per capsule may be determined my multiplying equation 2.9.5 by 2.9.6

BHK	baby hamster kidney
CHO	Chinese hamster ovary
CMP-NANA	Cytidine 5' –monophospho-N-acetyl neuraminic acid
CPP-s	Critical process parameters
EPO	erythropoietin
GFAT	glutamine:fructose-6-phosphate
GlcNAc	N-acetylglucosamine
GnT	N-acetylglucosamine transferase
GS	Glutamine synthase
HCG	human chorionic gonadotropin
HEK	human embryo kidney
IFN- γ	interferon- γ
IgG1	immunoglobulin-G1
Il-2	interlukin-2
L-GLN	L-glutamine
L-GLU	L-glutamate
NCAM	Neural adhesion molecule
NH ₄ Cl	ammonium chloride
OST	oligosaccharyl transferase
TCA	tricarboxylic acid
t-PA	tissue plasminogen activator
TNFR	tumour necrosis factor receptor
UDP-GNAc	Uradine Di Phosphate-N-acetylhexosamines
UDP-GalNAc	Uradine Di Phosphate-N-acetylgalactosamine
UDP-GlcNAc	Uradine Di Phosphate-N-acetylglucosamine

3.1: Introduction

The continued lack of rapid commercialisation of recombinant therapeutic drugs produced in mammalian cell cultures is attributed to the necessity to monitor and control a vast range of critical process parameters (CPP-s) which affect cell growth, productivity and product quality, namely protein glycosylation and/or aggregation. The CPP-s affecting high density Chinese hamster ovary (CHO) cell growth and large scale production of high quality recombinant protein drugs have been extensively reviewed in Chapter 1, and include

temperature, pH, levels of dissolved gases etc. Variations in the possible outcomes of altering culture parameters exist between different cell types (hybridoma, baby hamster kidney (BHK), CHO etc). Furthermore, different cell lines of the same cell type have been noted to respond differently to a changing culture condition.

It has been reported that the inefficient energy metabolism of mammalian cells in culture is still a major limiting factor for improvements in large scale high cell densities and recombinant protein production (Taschwer et al., 2012). Metabolism of mammalian cells is complex. Over the past three decades efforts have been made to understand the control and regulation of carbon source utilisation such as glucose and l-glutamine (L-GLN). It has been reported that cells in culture convert approximately 80 % of glucose to lactate (Zeng et al., 1998), irrespective of cell line. Such rapid aerobic glycolysis and glutaminolysis require the use of high concentrations of glucose and L-GLN in cultures resulting in an accumulation of subsequently toxic levels of the biosynthetic waste products lactate and ammonia.

L-GLN is an amino acid which may be rapidly used by cells that have high energy demands and synthesize large amounts of proteins and nucleic acids in addition to glucose as a carbon energy source. The metabolic fates of L-GLN in mammalian cells have been extensively documented and reviewed by Vriezen et al., (1997) and Schneider et al., (1996), as noted in the Introduction Chapter, Section 1.2.5. The theoretical yield of ammonia resulting from the catabolism of 1 mole of L-GLN is 2 moles. However due to the alternative possible pathways for both the amido group and amino group from L-GLN the actual yield is ammonia may be lower (Vriezen et al., 1997). It must also be considered that the fate of L-GLN nitrogen is dependent upon the cell line and concentration of other metabolites in the culture such as glucose and other amino acids. Mammalian cell lines are derived from different species and organs and so therefore differ in specific enzymatic and metabolic patterns, as reviewed by Schneider et al., (1996). An excess of L-GLN in cell culture medium undergoes irreversible chemical degradation to form pyrrolidonecarboxylic acid in aqueous solutions, such as cell culture media. For every one mole of L-GLN degraded, one mole of ammonia is released (Schneider et al., 1996), as described in the Introduction Chapter, Section 1.2.5. Typical concentrations of L-GLN in mammalian cell cultures vary in the range of 1-7 mM, depending on cell type and line, and typically yield concentrations up to 5 mM ammonia. (Freshney, 1987; Hayter et al., 1991). However there are reports of ammonia accumulation of up to 10 mM in a standard batch culture (Ozturk et al., 1992). In addition, initial L-GLN concentrations of 8-40 mM have also been reported, which in turn would result in the

accumulation of high ammonia concentrations (Flickinger et al., 1992). Efforts have been made in order to identify the optimum L-GLN concentration required for maximum cell growth and productivity, while maintaining the ammonia concentration accumulated in the culture below the critical level at which toxification is induced. Yang and Butler, (2000) demonstrated no significant difference in cell growth for CHO cells producing the recombinant protein erythropoietin (EPO) in the presence of 2 mM and 4 mM L-GLN. However the final yield of cells in 20 mM L-GLN was 50 % lower than that observed in 4 mM L-GLN. The highest recombinant protein concentration was present in the 2 mM L-GLN culture. Similar results were presented by Rajendra et al., (2000), in that the highest concentration of rIgG produced in CHO cell cultures occurred in the presence of 2 mM L-GLN. The effects of L-GLN limitation and an excess of L-GLN were investigated by Vriezen et al., (1997). In fed-batch cultures, it was determined that an L-GLN feed concentration of 0.5 to 2 mM resulted in a linear increase in biomass concentration for two murine hybridoma cell lines. No further increases in biomass yield were achieved when the concentration of L-GLN was in excess at 4 mM. Low L-GLN feed concentrations have also been noted to improve product yields. An L-GLN feed concentration of 0.3 mM enabled a 10-fold increase in the yield of interferon- γ (IFN- γ) in a CHO cell line. Although lower L-GLN concentrations appear to promote cell growth and productivity, reduced cellular growth rates and recombinant protein accumulation have been reported in cultures containing no glutamine (Burleigh et al., 2011; Rajendra et al., 2012) and cultures containing extremely limiting L-GLN concentrations, such as 0.1 mM (Wong et al., 2004).

As L-GLN has been presented above as one of the main energy and nitrogen sources for actively growing cells in culture, one may have previously hypothesised that increased concentrations of L-GLN in cultures would in turn promote higher biomass yields and subsequently increased recombinant protein production. In the above studies presented, it was concluded that the inhibitory effects of high L-GLN concentrations on cell growth and productivity, were due to an accumulation of a higher concentration of ammonia in the culture, in comparison to the levels obtained for low L-GLN concentrations. The accumulation of ammonia limits the growth and productivity enhancement possibilities of high L-GLN concentrations. Canning and Fields, (1983); Hansen and Emborg, (1994); Yang and Bulter, (2000) have all reported a negative effect of an ammonia concentration as low as 2 mM on cell growth and recombinant protein production.

Varying ammonia concentrations in cultures have also been noted to impact greatly on the quality (glycosylation) of the produced recombinant proteins, with many studies having reported on decreased sialylation levels caused by the accumulation of ammonia in culture media (Anderson and Goochee, 1995; Gawlitzek et al., 2000; Yang and Butler 2002). Anderson and Goochee, (1995) reported that ammonia levels as low as 2 mM resulted in overall decreased sialylation. At 50 mM ammonium chloride (NH₄Cl) there was a decrease in the relative percentage of disialylated forms in comparison to monosialylated forms. The presence of ammonia in the culture environment has also been reported to give rise to increased heterogeneity of glycoproteins resulting from an increase in antennarity of glycan structures (Gawlitzek et al., 1998; Grammatikos et al., 1998; Yang and Butler, 2000). Yang and Butler, (2002) investigated CHO cell cultures producing the recombinant protein EPO containing a concentration of 30 mM NH₄Cl. The accumulation of tetraantennary structures was reduced by 57 % in comparison to control cultures in which no ammonia was added. Gawlitzek et al., (2000) reported no significant difference in oligosaccharide branching for a CHO cell line producing the recombinant protein tumour necrosis factor receptor (TNFR-IgG). Variations in the effect of ammonia concentrations on production of the recombinant protein have also been noted to be cell line specific. Anderson and Goochee, (1995) showed that the secretion rate of a CHO cell line producing granulocyte colony-stimulating factor was not affected by an ammonia concentration of 0-25 mM NH₄Cl.

Two main mechanisms have been proposed to explain the relationship between ammonium toxicity and glycosylation, as reviewed in the Introduction Chapter, Section 1.3.4.2.2. One such mechanism explains how ammonium ions act as a weak base, which accumulates in the slightly acidic trans-golgi compartment, altering the pH outside of the range for optimal sialyl transferase activity (Anderson and Goochee, 1995; Gawlitzek et al., 2000). Another mechanism describes that elevated ammonium alters the balance of intracellular nucleotide sugar pools, resulting in increased levels of UDP-N-acetylhexosamines (UDP-GNAc) including UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-N-acetylgalactosamine (UDP-GalNAc) in a dose dependent manner (Gawlitzek et al., 1998; Yang and Butler, 2002).). Lower expression levels for the galactosylation and sialylation enzymes, β 1,4-galactosyltransferase and α 2,3-sialyltransferase, was observed under increased ammonium concentration conditions (Chen and Harcum, 2006).

In an effort to reduce the accumulation of ammonia in the culture media, a number of physical strategies have been employed, reviewed by Schneider et al., 1995 and noted in

Section 1.2.5 of Introduction Chapter. An alternative approach involved the substitution of L-GLN with less ammoniagenic substrates or analogs such as L-GLU, asparagines, and dipeptides such as l-alanyl-glutamine (commercially known as Glutamax) and glycyl-l-glutamine (Altamirano et al., 2004 Altamirano et al., 2000; Hassel et al., 1991). L-GLU in comparison to L-GLN has only one amino group and is utilised by cells much more efficiently than L-GLN resulting in lower accumulations of ammonia brought about by cell metabolism. L-GLU does not suffer from spontaneous decomposition into ammonium ions and so is a favourable substitute over glutamine as a carbon energy source (Altamirano et al., 2000).

A great deal of effort has been made to understand the impact of L-GLN, ammonia and L-GLN substitutes on mammalian cell growth, productivity and product quality over the past three decades. The results to date, do however suggest that the extent of the impact of the above metabolites may be cell line specific (Anderson and Goochee, 1995), with reports suggesting that CHO cells may be less sensitive to ammonia than other cell lines (Hassell et al., 1991). The impact of altering carbon and ammonia concentration on glycosylation has primarily focused on their effect on sialylation of glycoproteins, with only few papers reporting on their influence on other glycoforms, such as galactosylation (Hong et al., 2010). The authors of this study by Hong et al., (2010) suggested that the use of high ammonia concentrations, in the form of NH_4Cl , at the beginning of the culture, may impact the glycosylation profiles of recombinant proteins more adversely than that which may occur when high ammonia concentrations arise throughout the culture period as a result of cell metabolism/chemical degradation of L-GLN. In fitting with the overall first aim of the thesis, efforts will be made to identify L-GLN and ammonia as CPP-s affecting high cell densities, productivities and product quality with the overall aim of finding optimum medium concentrations of the metabolites for most efficient growth and productivity and quality. In doing so complete glycol profiles will be obtained using methods described by Royle et al., (2008) allowing a thorough analysis of effect of L-GLN, ammonia and L-GLN substitution on complete protein quality characterisation. Aggregation of protein will also be investigated. The experimental design will involve the inclusion of L-GLN concentrations in the range of 0, 4 and 12 mM, L-GLU concentrations in the range of 0-4 mM and ammonia concentrations (NH_4Cl) in the range of 5-15 mM. A concentration of 4 mM L-GLN is typical for the majority of mammalian cell culture process, according to medium manufacturer guidelines (Sigma Aldrich, 2012). In previous studies, mentioned above, it has been noted that higher

concentrations of L-GLN such as 40 mM have been previously tested for mammalian cell lines (Flickenger et al., 1992). However in this study 12 mM L-GLN was chosen for the highest concentration of L-GLN tested as the concentration of ammonia arising from its metabolism would be more comparable to those levels which typically arise in batch cultures (Ozturk et al., 1992). The 4 mM L-GLU concentration may be directly compared and contrasted to the 4 mM L-GLN culture enabling the determination of the possibility of this metabolite as a substitute for L-GLN. The rationale behind choosing 5-15 mM NH₄Cl is due to the fact that these are the concentrations of NH₄Cl typically incorporated into cell culture medium to test the effect of ammonia (Hong et al., 2010). To ensure that any growth characteristics displayed by the cells in the ammonia cultures are due to the impact of ammonia and not due to the absence of a carbon source, 4 mM L-GLN will also be included in these 5-15 mM NH₄Cl cultures. It is hypothesised that the results of such an experiment will enable (i) the determination of the effect of the varying concentrations of the above metabolites on this specific cell line, CHO DP-12 producing the recombinant protein Immunoglobulin G1 (IgG1) (ii) a complete and dynamic study of the effect of the above metabolites on the heterogeneity of a range of N-glycan structures, including galactose and sialic acid structures and (iii) the determination if previous reports investigating the effect of ammonia through the addition of high initial NH₄Cl concentration in the cultures, do over-estimate the impact of ammonia in comparison to high concentrations arising throughout the cultivation period. It is envisaged that comparison of results from the 12 mM L-GLN culture and 15 mM Ammonia culture will enable such comparisons.

3.2: Results

3.2.1: Effect on CHO cell growth and viability

To evaluate the effect of varying concentrations of L-GLN, L-GLU and ammonia on the CHO cell growth and viability, CHO cells at an initial concentration of 3×10^5 cells/ml were grown in ExCell CHO 325 PF media supplemented with 0-12 mM L-GLN, 0-4 mM L-GLU and 5 and 15 mM Ammonia. The Ammonia cultures also contained 4 mM L-GLN. The final culture volume was 300 ml and cultures were carried out in 1 L Erlenmeyer shake flasks in triplicate.

Figures 3.2.1.1 and 3.2.1.2 and the growth stats presented in Table 3.2.1.1 below, allow the growth patterns and viability of the cells in the various cultures to be compared and

contrasted. The highest maximum specific growth rate, μ_{\max} (h^{-1}), was displayed by cells cultured in media containing 4 mM L-GLN, 65 % higher than that observed in cultures containing no L-GLN. Increasing the concentration of L-GLN in the media to 12 mM resulted in a minimal decrease in the maximum specific growth rate displayed by the cells. However the maximum viable cell density attained in these cultures was not significantly different. The inclusion of 4 mM L-GLN and 12 mM L-GLN in the culture media resulted in an increased maximum viable cell density of 51 % and 42 %, respectively, in comparison to the 0 mM L-GLN culture. The viability remained high, above 96 %, until day 6 in the 4 mM L-GLN culture and until day 5 in the 12 mM L-GLN culture.

The lowest maximum specific growth rate was displayed by cells cultured in media containing 4 mM L-GLU. Neither the growth nor the maximum viable cell density displayed by the cells in the 4 mM L-GLU culture were significantly different from the 0 mM culture. Both the 4 mM L-GLU culture and the 0 mM culture displayed increased culture longevity in comparison to all other cultures. Viability in the 0 mM and 4 mM L-GLU cultures remained above 86 % until day 16 and above 96 % until day 11 respectively.

The inclusion of 5 mM Ammonia and 15 mM Ammonia in the culture media, already containing 4 mM L-GLN, resulted in a 38 % and 58 % lower maximum specific growth rate, respectively, in comparison to the 4 mM L-GLN culture. However the maximum specific growth rate displayed by the cells was higher than that of the 0 mM culture. Overall the lowest maximum viable cell density reached was in the 15 mM ammonia culture. The cells cultured in the presence of 15 mM Ammonia had a 60 % lower maximum viable cell density at the end of the exponential growth period in comparison to cells culture in 4 mM L-GLN. The inclusion of 5 and 15 mM ammonia in the culture media did not however appear to affect the viability of the cells. The viability of the cells appeared to follow the same trend as that observed in the 4 mM and 12 mM L-GLN cultures.

As noted in Figure 3.2.1.1 there were differences in the time points at which cells entered in the exponential phase, stationary phase and death phase between each of the culture conditions tested. For the remainder of the results to follow, comparisons will be made between concentrations of protein produced, quality of protein produced and metabolic consumption and production profiles over the various exponential and stationary growth periods. As there are differences in the time points when these specific growth periods occurred, Table 3.2.1.2 below presents the periods of time when the lag, exponential and stationary phases ended in each culture.

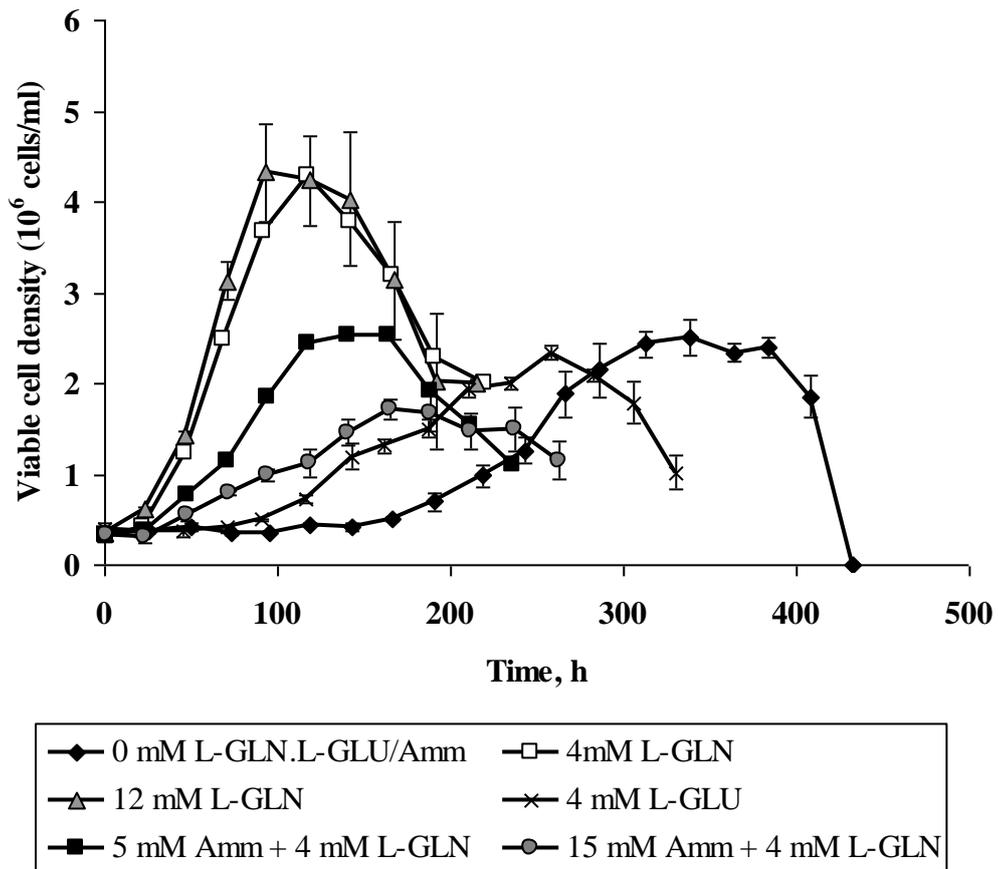


Figure 3.2.1.1: Correlation of the viable cell density displayed by cells over time when cultured in varying concentrations of L-GLN, L-GLU and ammonia. Cells were inoculated at 0.3×10^6 cell/ml into a culture volume of 300 ml ExCell CHO 325 PF media containing 0-12 mM GLN, 0-4 mM GLU and 5-15 mM Amm. Cultures were performed in 1 L Erlenmeyer shake flasks. Viable cell density was determined daily until viability reached below ~50 %. Values are means +/- STD of triplicate cultures performed for each condition tested.

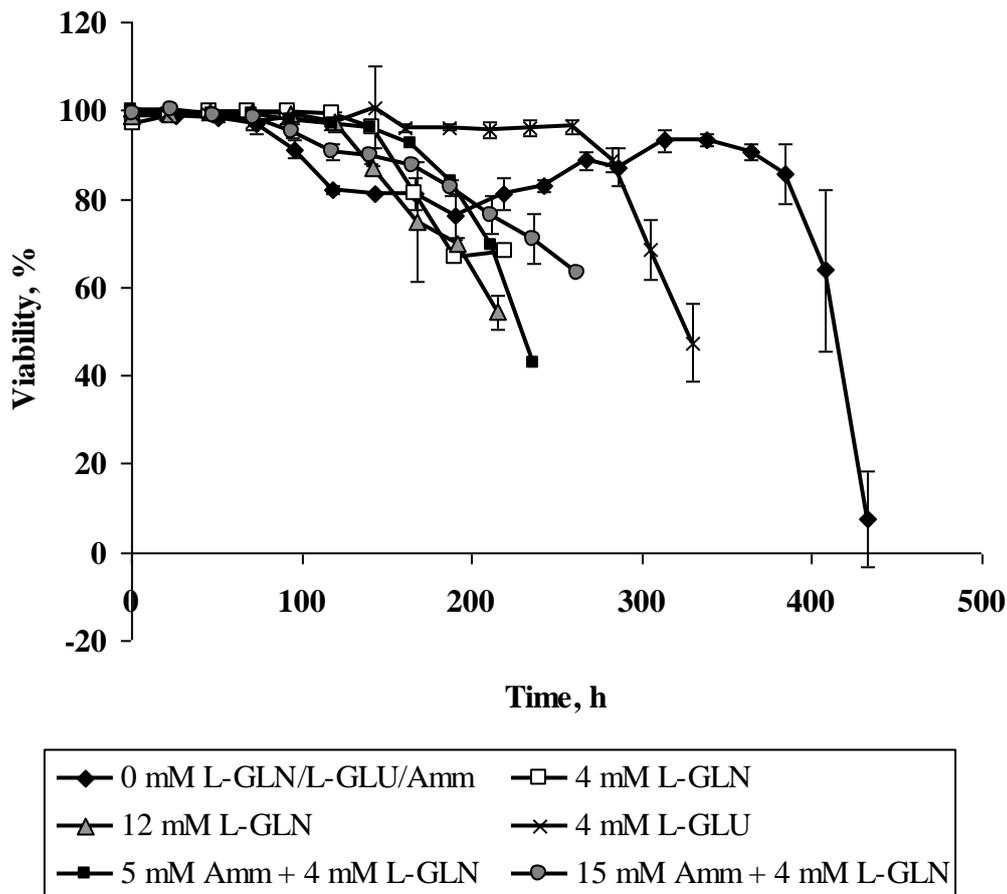


Figure 3.2.1.2: Correlation of the % viability over the cultivation period for cells grown in the presence of varying concentrations of GLN, GLU and Amm. Cell viability was determined on a daily basis until cultivation was ceased when viability decreased to ~50 %. Values are means +/- STD of triplicate cultures performed for each culture condition tested.

Table 3.2.1.1: Calculated maximum specific growth rates displayed by cells cultured in varying concentrations of L-GLN, L-GLU and ammonia over the exponential growth period. The growth rate was calculated from a plot of the logarithm of the viable cell density over the cultivation time (days) Values are mean of triplicate cultures performed for each of the conditions tested +/- STD.

Culture	μ_{max} (h ⁻¹)	Max VCD (*10 ⁶ cells/ml)
0 mM L-GLN/L-GLU/Amm	0.0107 +/- 0.0005	2.425 +/- 0.075
4 mM L-GLN	0.0312 +/- 0.0006	4.295 +/- 0.191
12 mM L-GLN	0.0282 +/- 0.0005	4.222 +/- 0.371
4 mM L-GLU	0.0096 +/- 0.0002	2.337 +/- 0.085
5 mM Amm + 4 mM L-GLN	0.0195 +/- 0.0015	2.49 +/- 0.240
15 mM Amm + 4 mM L-GLN	0.0130 +/- 0.0002	1.715 +/- 0.120

Table 3.2.1.2: Time point (day) at which each corresponding growth phase ended in each of the culture conditions tested as determined from an LN plot of viable cells density ($\times 10^6$ cells/ml) over the cultivation period.

Culture	End of lag phase	End of exponential phase	End of stationary phase
0 mM L-GLN/L-GLU/Amm	5	13	17
4 mM L-GLN	1	4	7
12 mM L-GLN	no lag phase	4	7
4 mM L-GLU	3	11	13
5 mM Amm + 4 mM L-GLN	1	5	7
15 mM Amm + 4 mM L-GLN	1	6	10

3.2.2 Effect on cell metabolism

The concentration of the major carbon sources, glutamine and glucose, and their corresponding by-products, ammonia and lactate, were measured in cultures supplemented with GLN, GLU and AMM (Figures 3.2.2.1-3.2.2.4). Samples were removed from cultures on a daily basis and centrifuged at 200 g for 5 minutes, followed by filtration of the supernatant (0.22 μ m PTFE) in order to remove cells and culture debris. Glucose and lactate concentrations were determined using HPLC analysis, and glutamine and ammonia analysis was carried out using commercially available enzymatic assays kits, as described in Materials and Methods section 2.3.3. Determination of the concentration of the various metabolites enabled the calculation of the specific substrate uptake rates for each of the experimental cultures (Tables 3.2.2.1 and 3.2.2.2).

Note: no specific production rates were calculated for ammonia for the 0 mM L-GLN/L-GLU/Amm culture and 4 mM L-GLU as the concentration of ammonia remained at an almost constant levels ranging between 1-1.6 mM for the 0 mM L-GLN/L-GLU/Amm culture and between 1-1.3 mM for the 4 mM L-GLU culture.

All cultures were performed in ExCell CHO 325 PF media supplemented with varying concentrations of L-GLN, L-GLU and ammonia. The concentration of glucose in the commercially available media is expected to be 17.4 mM. In cultures containing 4 mM and 12 mM L-GLN, all glucose was consumed at the end of the stationary phase of growth. There was no difference portrayed in the relative specific consumption rates of glucose between the two culture conditions. The specific production of lactate was similar for both culture conditions, with similar yields of lactate from glucose reported for both culture conditions.

The absence of L-GLN/L-GLU/Ammonia from cultures resulted in the consumption of glucose by the end of the exponential growth period. However the specific rate of glucose consumption was similar to that displayed by cells cultured in the presence of 4 and 12 mM L-GLN. A 2-fold increase in the specific lactate production rate was observed for the 0 mM L-GLN/L-GLU/Ammonia culture in comparison to the 4 mM L-GN culture. A yield of 1.39 mmol/mmol of lactate on glucose was determined over the culture period in the 0 mM L-GLN/L-GLU/Ammonia culture, in comparison to a yield of 0.95 mmol/mol, which was determined for the 4 mM L-GLN culture. The results suggest a less favourable utilisation of glucose, via the glycolytic pathway, by the cells resulting in increased lactate production. The specific glucose consumption rate displayed by the cells cultured in 4 mM L-GLU was 40 % and 33 % lower than that observed in the 0 mM L-GLN/L-GLU/Ammonia and 4 mM L-GLN cultures respectively. No significant difference in the specific lactate production rate was observed in comparison to the 0 mM L-GLN/L-GLU/Ammonia culture and the overall yield of lactate on glucose was similar to that observed in the 0 mM L-GLN/L-GLU/Ammonia culture, 1.35 mmol/mmol. Such results again signify inefficient glucose metabolism when glutamine is replaced by glutamate. There was no significant difference in the glucose consumption rate displayed by cells cultured in 5-15 mM ammonia, also containing 4 mM L-GLN, in comparison to the 4 mM L-GLN culture. There was however a significant difference in the specific lactate production rate observed in the 15 mM ammonia culture in comparison to the 5 mM Ammonia culture and the 4 mM L-GLN culture. This did not however result in an increased accumulation of lactate due to the lower number of cells present producing the lactate. Glucose was not depleted in the 5 mM Ammonia culture until day 10, 3 days after cells left stationary growth phase. A concentration of 0.5 mM glucose was present in the 15 mM Ammonia culture at the end of the culture period, 2 days after the cells had left the stationary phase.

A 65 % higher specific glutamine consumption rate was noted in the 12 mM L-GLN culture, in comparison to the 4 mM L-GLN culture. L-GLN was depleted at the end of the exponential phase in the 4 mM L-GLN cultures. It was not however depleted at any stage in the 12 mM L-GLN cultures. The growth of cells was limited in the 4 mM L-GLN culture by L-GLN. As expected increased ammonia production rates were noted in the 12 mM L-GLN culture, in comparison to the 4 mM L-GLN culture. As both the rates of L-GLN consumption and ammonia production increased proportionally in the 12 mM L-GLN culture in comparison to the 4 mM L-GLN culture, it would be expected that the yield of ammonia

from L-GLN would be similar for both culture conditions. This was the case with yields of ammonia from L-GLN determined as 1.07 and 1.05 mmol/mmol for the 4 mM L-GLN and 12 mM L-GLN cultures, respectively. Both the absence of L-GLN and its replacement by L-GLU proved beneficial in reducing the accumulation of ammonia in the cultures. The concentration of ammonia remained below 1.6 mM and 1.3 mM during exponential growth for both the 0 mM L-GLN/L-GLU/Ammonia and the 4 mM L-GLU cultures. In the ammonia cultures (5 & 15 mM), which had an initial L-GLN concentration of 4 mM, L-GLN was not consumed at the end of the exponential growth phase. It was consumed before the end of the stationary phase in the 5 mM Ammonia culture. Similar specific L-GLN consumption rates were displayed in the 5 and 15 mM Ammonia cultures and were approximately 52 % higher than those displayed in the 4 mM L-GLN culture. The increased specific L-GLN consumption rates did not result in an accumulation of ammonia. The cell specific ammonia production rate in the 5 and 15 mM ammonia cultures was lower than that observed in the 4 mM L-GLN cultures. The lower specific production rate combined with the lower number of cells present in the ammonia cultures resulted in an ammonia accumulation of only ~ 2 mM higher than the concentration which was present initially in the media. The lower levels of ammonia production in these two cultures resulted in expected low yields of ammonia from L-GLN, determined to be 0.64 and 0.63 for the 5 mM and 15 mM ammonia cultures, respectively.

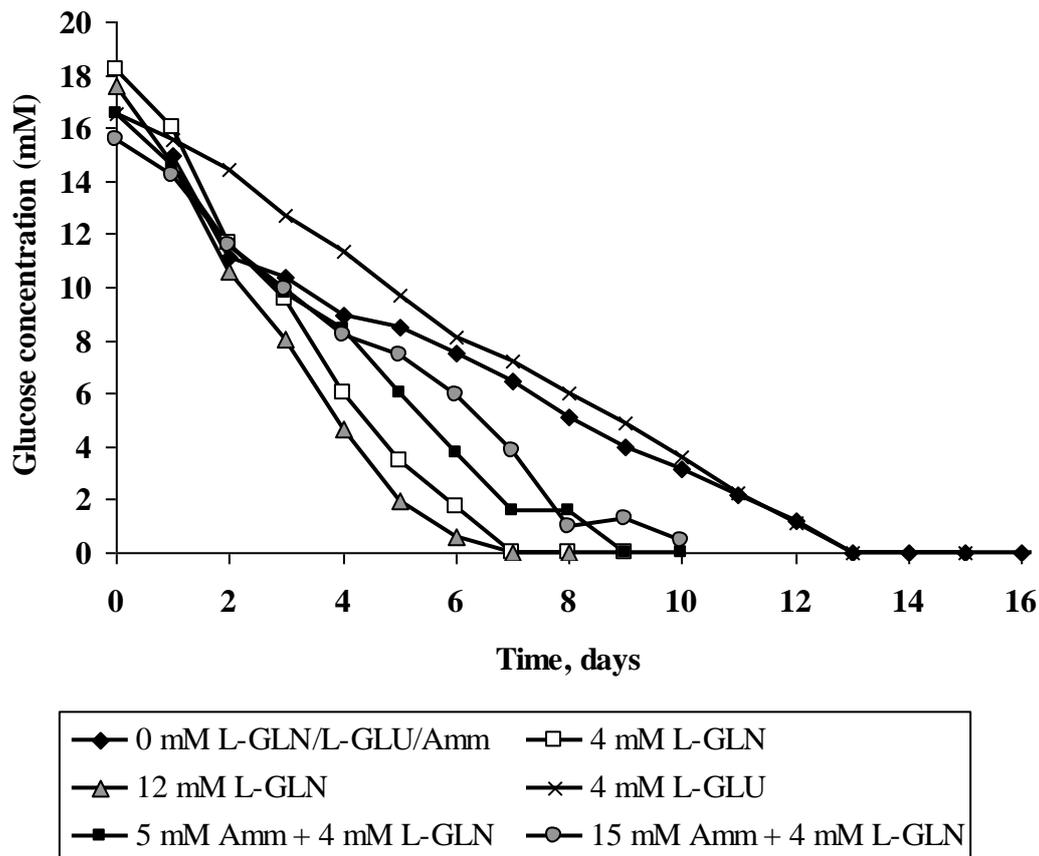


Figure 3.2.2.1: Concentration of glucose present in cultures containing varying concentrations of L-GLN, L-GLU and ammonia over the duration of the respective cultivation periods. The concentrations of glucose were determined offline by HPLC analysis using Supelco H column. Values are mean of triplicate cultures performed in parallel, +/- STD, for each of the culture conditions tested.

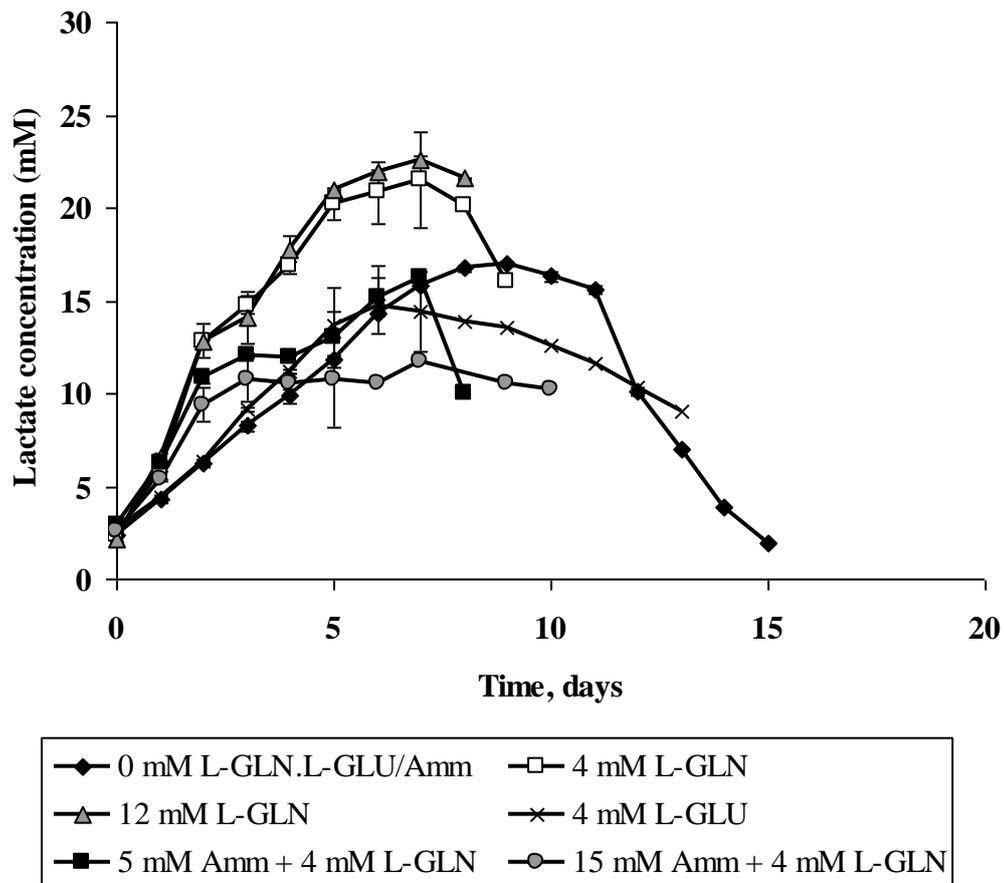


Figure 3.2.2.2: Concentration of lactate present in cultures containing varying concentrations of L-GLN, L-GLU and ammonia over the duration of the respective cultivation periods. The concentrations of lactate were determined offline by HPLC analysis using Supelco H column. Values are mean of triplicate cultures performed in parallel, +/- STD, for each of the culture conditions tested.

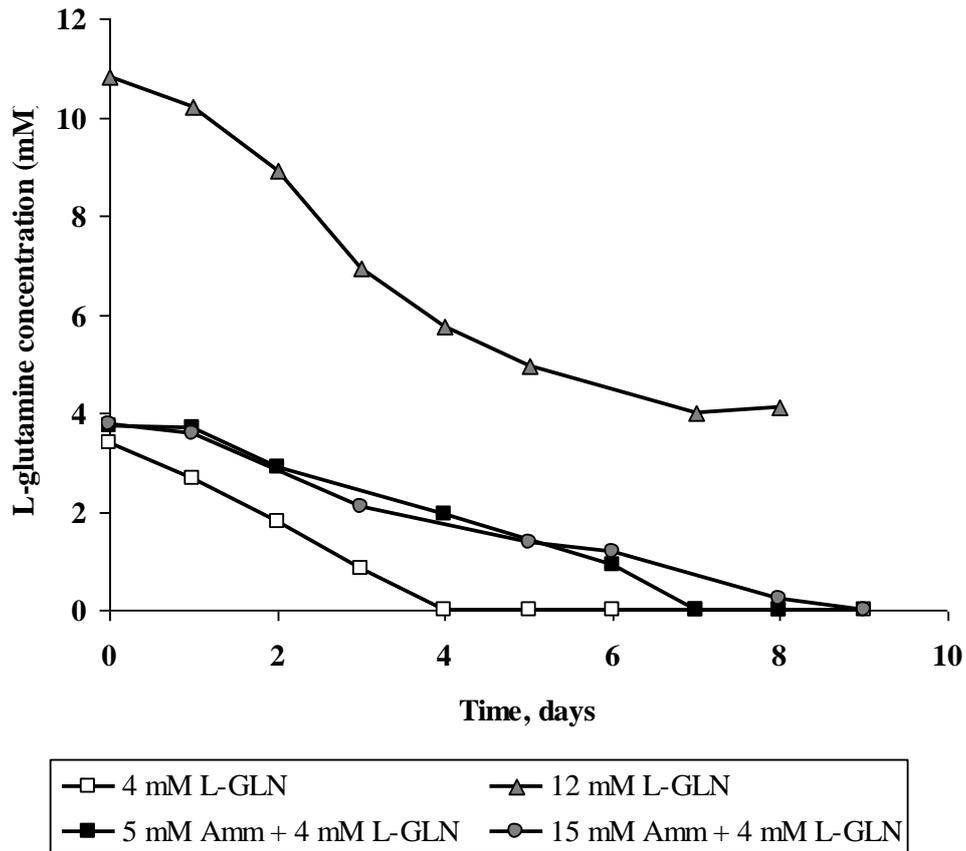


Figure 3.2.2.3: Concentration of glutamine present in cultures containing varying concentrations of L-GLN, L-GLU and ammonia over the duration of the respective cultivation periods. The concentrations of glutamine were determined offline using a commercially available enzymatic assay kit. Values are mean of triplicate cultures performed in parallel, +/- STD, for each of the culture conditions tested.

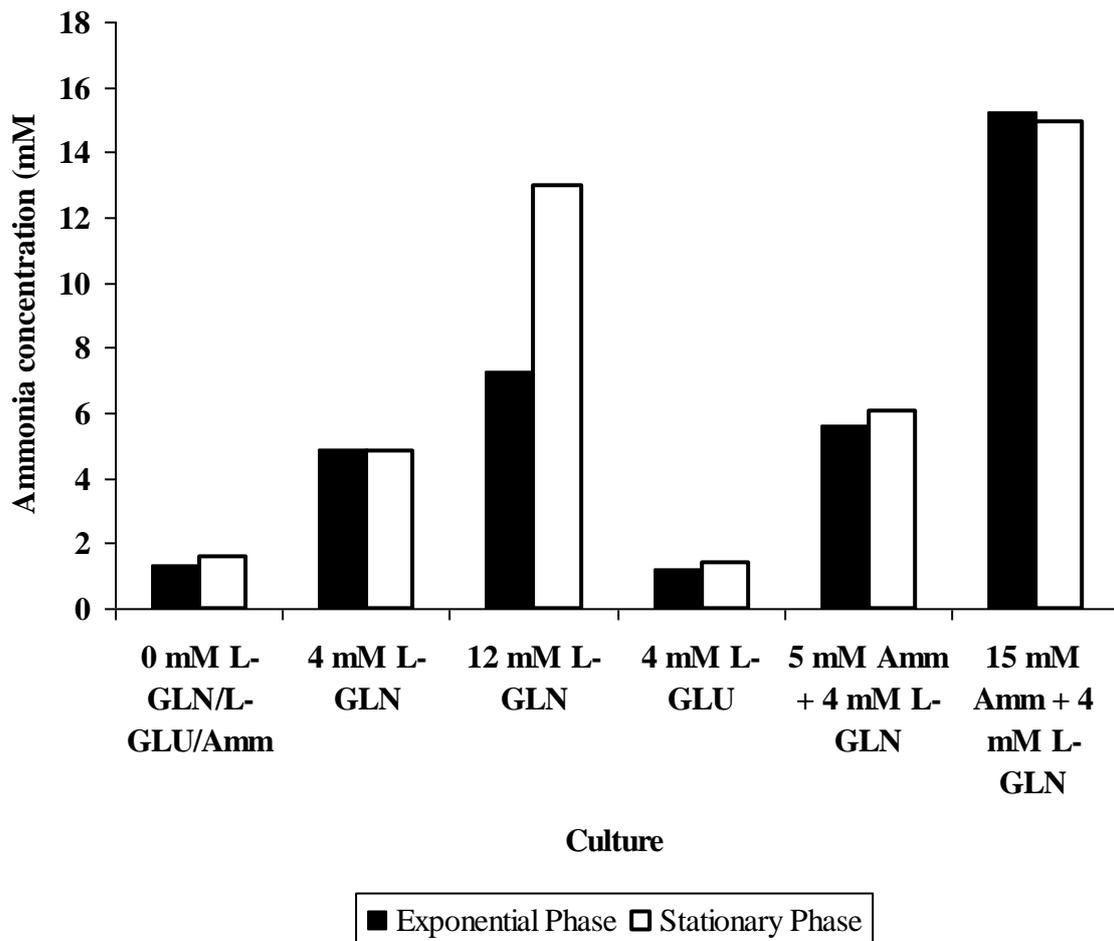


Figure 3.2.2.4: Concentration of ammonia present in cultures containing varying concentrations of L-GLN, L-GLU and ammonia at the end of the exponential and stationary culture periods. The concentrations of ammonia were determined offline using a commercially available enzymatic assay kit. Values are mean of triplicate cultures performed in parallel for each of the culture conditions tested.

Table: 3.2.2.1: Calculated rates of consumption and production of glucose and lactate as determined by offline analysis throughout the culture period. Cell specific consumption and production rates of the key metabolites were determined during the exponential growth phase. Values are mean of triplicate cultures performed in parallel, +/- STD, for each of the culture conditions tested.

	Glucose consumption rate (mM/day)	Lactate production rate (mM/day)	q_{glucose} (mmoles/10⁶ cell/day)	q_{lactate} (mmoles/10⁶ cell/day)
0 mM L-GLN/L-GLU/Amm	0.99 +/- 0.07	1.86 +/- 0.007	$9.27 * 10^{-4}$ +/- $2.82 * 10^{-5}$	$2.32 * 10^{-3}$ +/- $5.62 * 10^{-5}$
4 mM L-GLN	3.19 +/- 0.003	3.49 +/- 0.17	$8.3 * 10^{-4}$ +/- $5.23 * 10^{-5}$	$1.1 * 10^{-3}$ +/- $8.59 * 10^{-5}$
12 mM L-GLN	3.28 +/- 0.18	3.65 +/- 0.30	$7.7 * 10^{-4}$ +/- $9.52 * 10^{-5}$	$1 * 10^{-3}$ +/- $1.5 * 10^{-4}$
4 mM L-GLU	1.33 +/- 0.12	2.35 +/- 0.25	$5.6 * 10^{-4}$ +/- $4.86 * 10^{-5}$	$2.7 * 10^{-3}$ +/- $7 * 10^{-4}$
5 mM Amm + 4 mM L-GLN	2.03 +/- 0.16	2.94 +/- 0.03	$8 * 10^{-4}$ +/- $7.88 * 10^{-5}$	$1.1 * 10^{-3}$ +/- $2 * 10^{-4}$
15 mM Amm + 4 mM L-GLN	1.59 +/- 0.02	2.25 +/- 0.13	$1.1 * 10^{-3}$ +/- $1 * 10^{-4}$	$2.4 * 10^{-3}$ +/- $4.92 * 10^{-6}$

Table: 3.2.2.2: Calculated rates of consumption and production of glutamine and ammonia as determined by offline analysis throughout the culture period. Cell specific consumption and production rates of the key metabolites were determined during the exponential growth phase. Values are mean of triplicate cultures performed in parallel, +/- STD, for each of the culture conditions tested.

	Glutamine consumption rate (mM/day)	Ammonia production rate (mM/day)	$q_{\text{glutamine}}$ (mmoles/10⁶ cell/day)	q_{ammonia} (mmoles/10⁶ cell/day)
4 mM L-GLN	0.89	0.9	$2.26 * 10^{-4}$	$3.11 * 10^{-4}$
12 mM L-GLN	1.34	1.66	$3.84 * 10^{-4}$	$4.81 * 10^{-4}$
5 mM Amm + 4 mM L-GLN	0.86	0.814	$4.79 * 10^{-4}$	$3.24 * 10^{-4}$
15 mM Amm + 4 mM L-GLN	0.47	0.567	$4.99 * 10^{-4}$	$3.2 * 10^{-4}$

Table 3.2.2.3: Calculated yield of mmoles of by-product produced per mmoles of metabolites consumed over the culture period in cultures containing varying concentrations of L-GLN, L-GLU and ammonia. Values are mean of triplicate cultures performed in parallel, +/- STD, for each of the culture conditions tested.

	$Y_{\text{lactate/glucose}}$ (mmol/mmol)	$Y_{\text{ammonia/glutamine}}$ (mmol/mmol)
0 mM L-GLN	1.39 +/- 0.07	NA
4 mM L-GLN	0.95 +/- 0.11	1.07
12 mM L-GLN	1.04 +/- 0.06	1.05
4 mM L-GLU	1.35 +/-0.03	NA
5 mM Amm + 4 mM L-GLN	0.98 +/- 0.09	0.64
15 mM Amm + 4 mM L-GLN	0.92 +/-0.18	0.63

3.2.3 Effect on rIgG1 production

To evaluate the effect of varying concentration of GLN, GLU and ammonia in culture media on rIgG1 production, samples were removed from the cultures on a daily basis from day 4 onwards. As explained in Section 2.3.6 of the materials and methods section, rIgG1 was purified from media samples removed from the cultures prior to centrifugation at 200 g for 5 minutes to remove cells. rIgG1 present in the media was purified using Protein A/G Nab spin columns and quantified using the nanodrop at A 280 nm.

Figure 3.2.3.1 allows comparison of the quantities of rIgG1 produced in all cultures at the end of the exponential and stationary growth phases respectively. The highest concentration of rIgG1 present at the end of the exponential and stationary growth phases was present in the 4 mM L-GLU culture. The concentration of rIgG1 present at the end of the stationary phase was 50 %, 21 % and 28 % higher than that present in the 0 mM, 4 mM L-GLN and 12 mM L-GLN cultures respectively. There did not appear to be a significant difference in the concentration of rIgG1 produced in the 4 mM L-GLN and 12 mM L-GLN cultures. Both contained ~30 % higher concentrations of rIgG1 than the 0 mM culture. The lowest quantities of rIgG1 produced were in the 5 mM ammonia and 0 mM culture. The 15 mM ammonia culture contained a 20 % higher rIgG1 concentration in comparison to the 5 mM ammonia culture at the end of the stationary phase of growth.

The above variations in the concentrations rIgG1 produced in the above cultures are due to variations in the culture longevities as opposed to differences in the specific rIgG1 rates displayed by the cells. Table 3.2.3.1 summaries the rIgG1 production rates and the specific production rates, q_{IgG1} , displayed by the cells in the various media. All cultures, with the exception of the 0 mM culture, displayed the same specific rIgG1 productivities. It may therefore be proposed that the 4 mM L-GLU culture contained the highest concentration of rIgG1 due to a cultivation period of 13 days (including exponential and stationary growth phases). In regards to efficiency, the 4 mM L-GLN was more beneficial in that the concentration of IgG1 was only 18 % lower at the end of the culture period on day 8, in comparison to the maximum concentration reached in the 4 mM L-GLU culture on day 13.

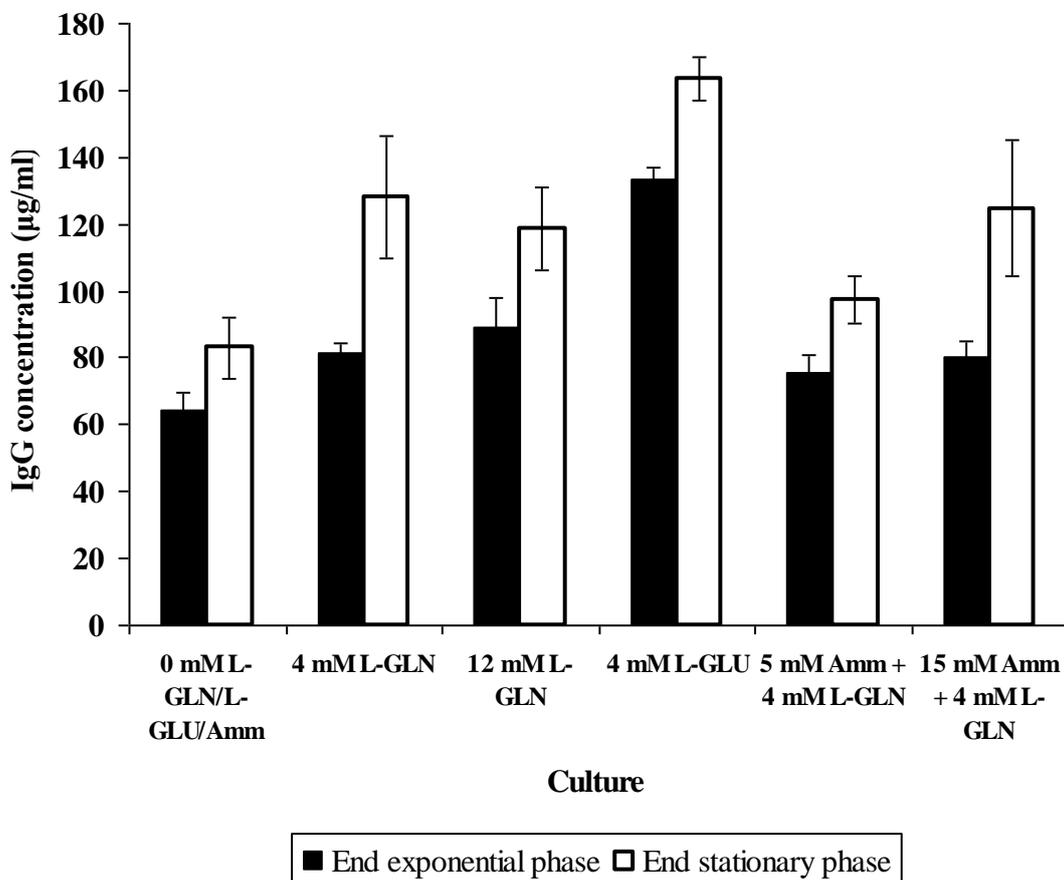


Figure 3.2.3.1: Concentration of rIgG1 measured at the end of the exponential and stationary growth phases in cultures containing varying concentrations of L-GLN, L-GLU and ammonia. rIgG1 was first purified using Protein A/G spin columns and quantified in duplicate using nanodrop A280 nm with an average value being recorded. Values are the mean of triplicate cultures performed in parallel, +/- STD, for each of the culture conditions tested.

Table 3.2.3.1: Calculated rIgG1 production rates and cell specific production rates for cells cultured in varying concentrations of L-GLN, L-GLU and ammonia. rIgG1 was first purified using Protein A/G spin columns and quantified using nanodrop A280 nm. Purified rIgG1 was quantified in duplicate, with an average value being recorded. IgG1 production rates were calculated from the time point at which protein was harvested until cessation of the culture period. Values presented above are the mean of triplicate cultures performed in parallel for each of the culture conditions tested.

Culture	IgG1 production rate $\mu\text{g/ml/h}$	q_{IgG1} $\mu\text{g}/10^6 \text{ cells/h}$
0 mM L-GLN/L-GLU/Amm	0.2694	0.1 +/- 0.025
4 mM L-GLN	0.61	0.217 +/- 0.029
12 mM L-GLN	0.3997	0.220 +/- 0.005
4 mM L-GLU	0.7291	0.266 +/- 0.014
5 mM Amm + 4 mM L-GLN	0.3301	0.250 +/- 0.02
15 mM Amm + 4 mM L-GLN	0.5953	0.278 +/- 0.014

3.2.4 Effect on rIgG1 quality

To assess the affect of varying concentrations of L-GLN, L-GLU and ammonia on the quality of rIgG1 produced, both glycan and aggregation analysis were completed on the purified rIgG1 samples harvested cultures at both the end of the exponential and stationary growth phases. The samples harvested were subjected to N-glycan release and labelled as outline in Materials and Methods section 2.3.7.1. HILIC characterisation of the released and labelled N-glycans produced a chromatogram consisting of 8 distinctly separated peaks. Each of the peaks was assigned a distinctive glucose units (GU) value by comparison to the retentions times of the standard A-AB labelled external dextran. Examination of exoglycosidase treated samples allowed for the corresponding number and linkage of the component monosaccharide units represented by each GU value to be elucidated, as described in Materials and Methods section 2.3.7.3. Aggregation analysis of the purified samples was carried out using size exclusion chromatography (SEC)

Figure 3.2.4.1 below illustrates the relative % of the different glycans present on rIgG1 samples harvested from the cultures at the end of the exponential growth phase. The results presented below are similar to that observed at the end of the stationary growth phase (results not shown). Irrelevant of the concentration of L-GLN, L-GLU or ammonia, all cultures displayed a similar trend in the relative % of glycans present. The earliest form of N-glycosylation of rIgG1 is denoted by M5, F6A1/A2 and F6A2 as described in Materials and

methods section 2.3.7.3. The highest relative percentage of glycans present on the rIgG1 samples were the early glycoforms, predominantly F6A2. The galactosylated glycoforms are denoted by F(6)A2[6]G(4)1 and F(6)A2[3]G(4)1, which contain 1 galactose unit on either of the outer GlcNAc arms, and F(6)A2G2 and A2G2 which contain a galactose residue on each of the outer GlcNAc arms. Of the galactosylated glycoforms present, the F(6)A2[6]G(4)1 were the predominant form in all cultures, independent of supplement concentration. The glycan which was of lowest relative % in the cultures was the most complex fucosylated and sialyated glycan F(6)A2G2S1

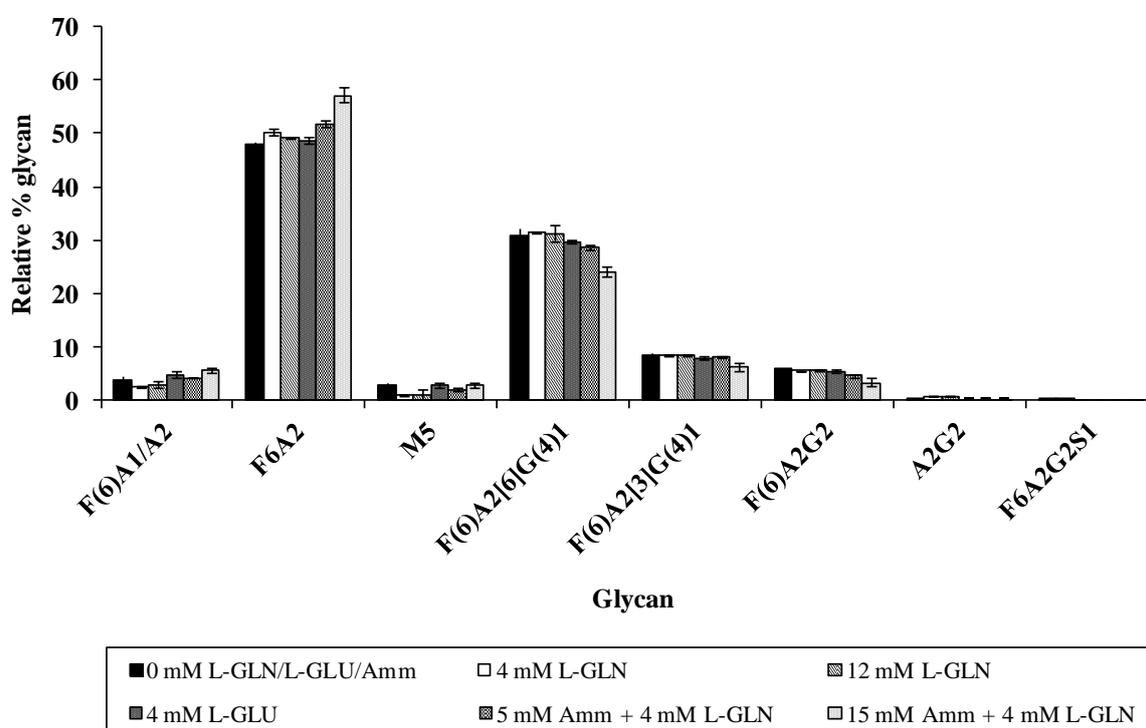


Figure 3.2.4.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from cultures containing varying concentrations of L-GLN, L-GLU and Ammonia at the end of the exponential growth phase. N-glycans were released from the rIgG1 samples by means of PNGaseF digestion. Samples for HILIC analysis were labeled by adding 5 μ l of 2AB labelling solution. The retention time of the glycan peaks was compared with an external A-AB labelled dextran standard for structural elucidation by converting the retention time of the peaks into GU. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for each condition tested.

It is evident that there was no significant difference in the trends of glycoforms identified on rIgG1 harvested from each of the different cultures. However a more in depth evaluation of the relative % of each type of glycoform present does indicate significant differences in the levels of glycosylation between cultures. Figure 3.2.4.2 displays the relative % of the F(6)A2 glycoform present on purified rIgG1 samples harvested from cultures at the end of the exponential phase of growth. For this early glycoform type, the highest relative % occurred in the 15 mM ammonia culture. Figure 3.2.4.3 shows the relative % of M5, another early glycoform, present on rIgG1 harvested in each of the cultures at the end of the exponential phase. Similarly, the highest relative % of this glycoform occurred in the 15 mM ammonia culture. The lowest relative % occurred in the cultures containing L-GLN, independent of the L-GLN concentration present. The relative % was 65 % lower for the 4 mM L-GLN culture and 60 % lower for the 12 mM L-GLN culture in comparison to the 0 mM culture. Although the 5 mM and 15 mM Ammonia cultures did contain 4 mM L-GLN, the relative % of M5 glycans present was significantly higher than the L-GLN cultures. The relative % of M5 glycans present on rIgG1 samples in 15 mM ammonia cultures was 30 % higher than the 5 mM ammonia culture. Similar results were obtained from glycan analysis of rIgG1 purified at the end of the stationary phase in cultures (results not shown).

M5 is the earliest glycoform resulting from N-glycosylation of rIgG1. It arises as a result of early glycan trimming in the Endoplasmic Reticulum (ER) membrane. The lower levels of M5 present on rIgG1 samples harvested from cultures containing 4 mM and 12 mM L-GLN indicate that a higher level of complex type glycan processing took place in these cultures. The higher relative % of M5 present on rIgG1 purified from the ammonia cultures demonstrates the impact of high initial ammonia concentrations on complex glycan processing. The relative % of M5 present in 4mM L-GLU cultures was similar to that of the 0 mM cultures. The substitution of GLN with GLU does not achieve the same level of complex glycan processing.

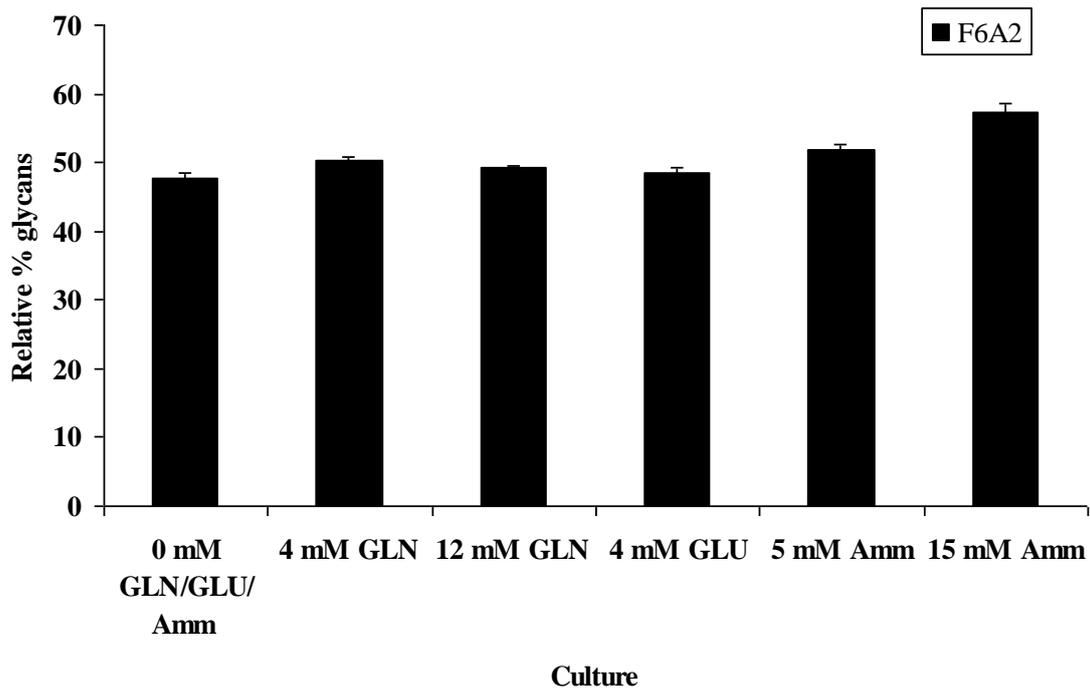


Figure 3.2.4.2: Relative % of F(6)A2 glycan forms detected on rIgG1 samples harvested from cultures containing varying concentration of L-GLN, L-GLU and ammonia. F(6)A1/A2 was detected using HILIC following PNGaseF release and 2-AB labelling. The results above are based on the analysis of samples harvested at the end of the stationary growth period for each culture condition tested. Results are directly comparable to those found for the analysis of samples from the end of the exponential growth period. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for each condition tested +/- STD.

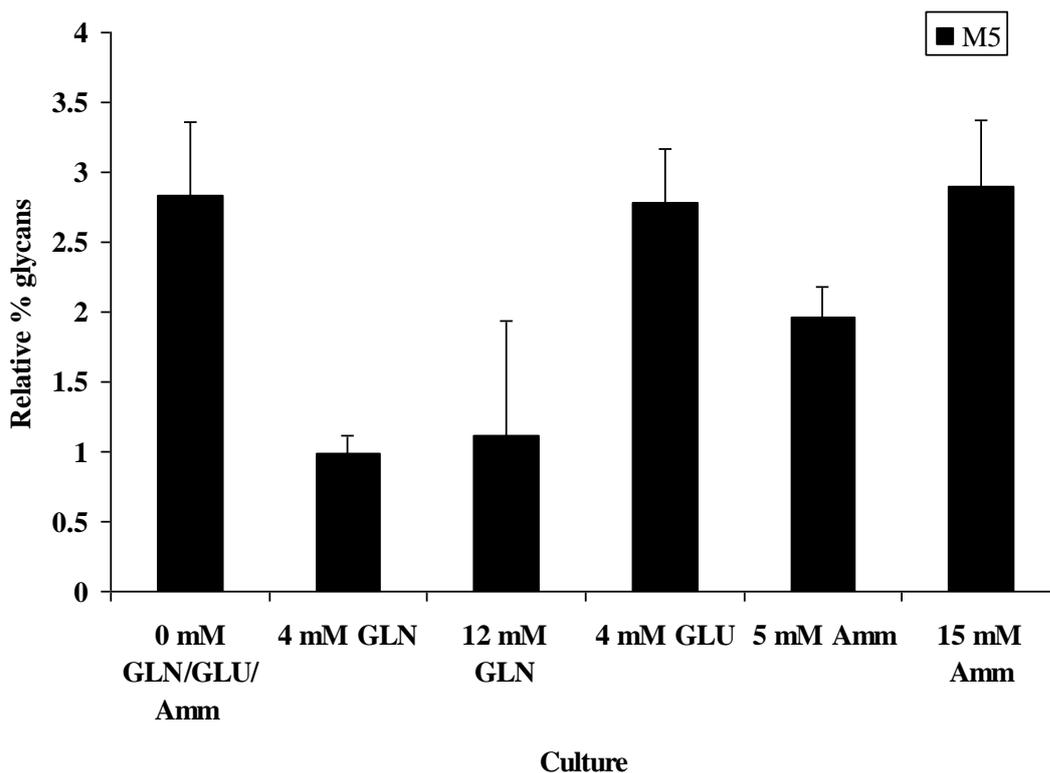


Figure 3.2.4.3: Relative % of M5 glycan forms detected on rIgG1 samples harvested from cultures containing varying concentration of L-GLN, L-GLU and ammonia. M5 was detected using HILIC following PNGaseF release and 2-AB labelling. The results above are based on the analysis of samples harvested at the end of the stationary growth period for each culture condition tested. Results are directly comparable to those found for the analysis of samples from the end of the exponential growth period. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for each condition tested +/- STD.

In order to determine if complex glycan processing is dependent on the presence of GLN in the culture and affected by high initial ammonia concentrations, it is necessary to compare and contrast the relative % of complex type glycans present on rIgG1 harvested from each of the cultures. Complex processing of glycans may result in an array of glycoforms containing varying levels of GlcNAc, galactose and sialic acid residues.

Figures 3.2.4.4 and 3.2.4.5 illustrate the relative % of glycoforms containing 1 galactose residue attached to either of one of the GlcNAc outer arms. There was no significant difference in the relative % of these two glycoforms between the cultures, with the exception of the 15 mM Ammonia culture. The relative % of F(6)A2[6]G(4)1 and F(6)A2[3]G(4)1 were

21 % and 16 % lower in the 15 mM ammonia cultures in comparison to the 0 mM culture. Figure 3.2.4.6 shows the relative % of fucosylated glycans containing 2 galactose residues attached to the GlcNAc outer arms. For this glycoform a 44 % decrease in the relative % was noted in the 15 mM ammonia culture in comparison to the 0 mM culture. Of the A2G2 glycoform again the lowest relative % of this glycan occurred in the 15 mM ammonia culture. However there was no significant difference in comparison to the culture containing 0 mM L-GLN. These results do indicate that complex glycan processing is affected by high ammonia concentrations. However the culturing of cells in varying concentrations of GLN did not appear to influence the galactosylation of glycans in comparison to cultures containing no L-GLN and cultures containing L-GLU.

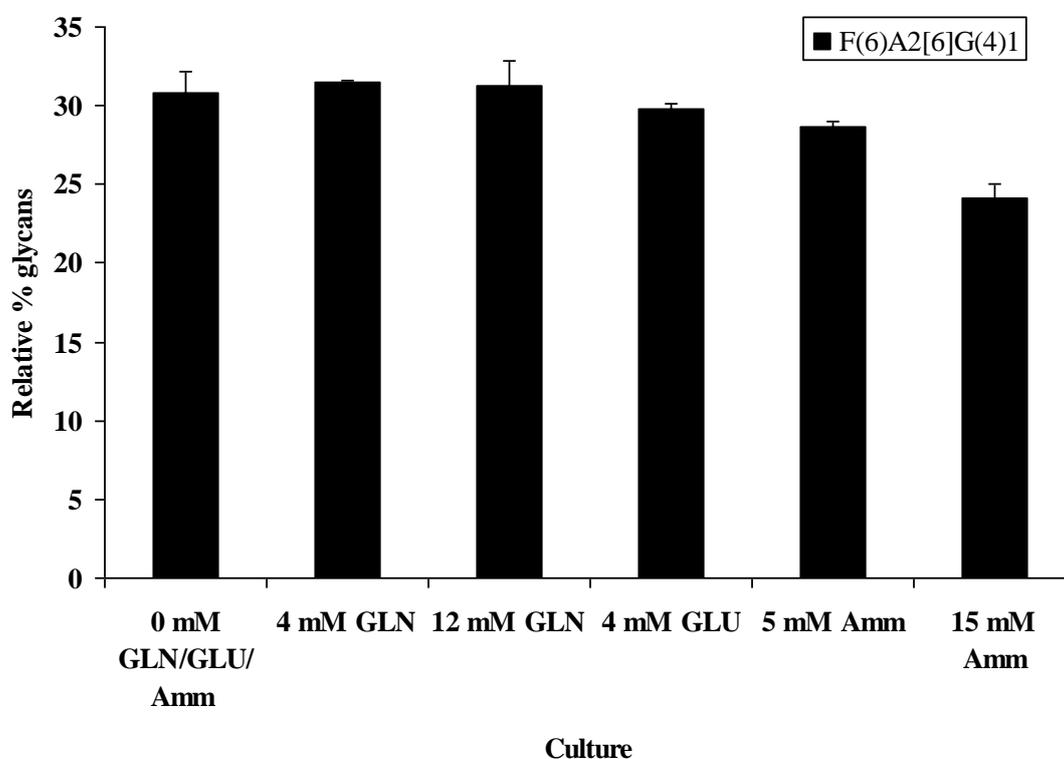


Figure 3.2.4.4: Relative % of F(6)A2[6]G(4)1 glycan forms detected on rIgG1 samples harvested from cultures containing varying concentration of L-GLN, L-GLU and ammonia. F(6)A2[6]G(4)1 was detected using HILIC following PNGaseF release and 2-AB labelling. The results above are based on the analysis of samples harvested at the end of the stationary growth period for each culture condition tested. Results are directly comparable to those found for the analysis of samples from the end of the exponential growth period. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for each condition tested +/- STD.

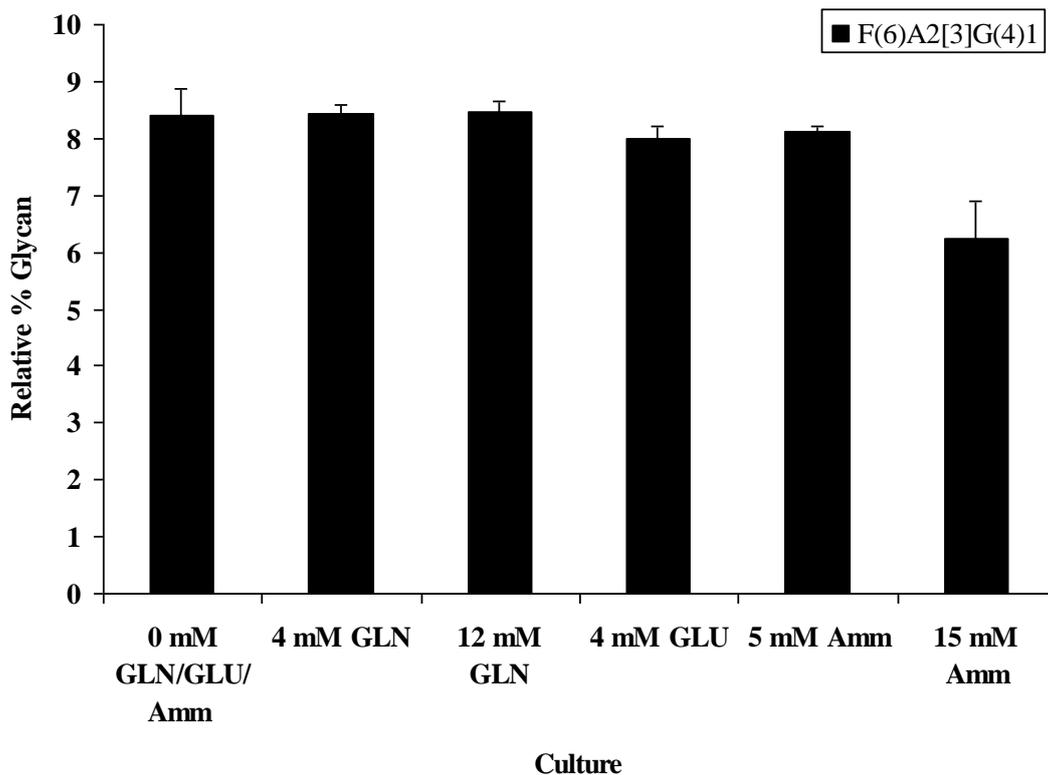


Figure 3.2.4.5: Relative % of F(6)A2[3]G(4)1 glycan forms detected on rIgG1 samples harvested from cultures containing varying concentration of L-GLN, L-GLU and ammonia. F(6)A2[3]G(4)1 was detected using HILIC following PNGaseF release and 2-AB labelling. The results above are based on the analysis of samples harvested at the end of the stationary growth period for each culture condition tested. Results are directly comparable results to those found for the analysis of samples from the end of the exponential growth period. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for each condition tested +/- STD.

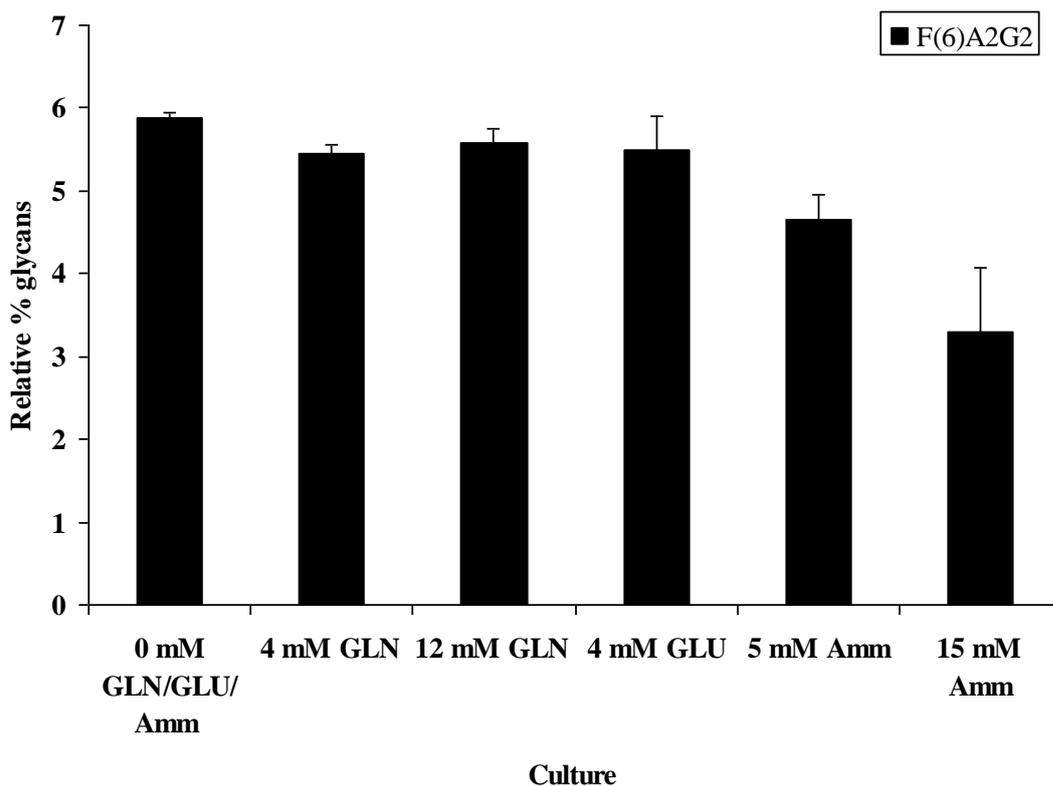


Figure 3.2.4.6: Relative % of F(6)A2G2 glycan forms detected on rIgG1 samples harvested from cultures containing varying concentration of L-GLN, L-GLU and ammonia. F(6)A2G2 was detected using HILIC following PNGaseF release and 2-AB labelling. The results above are based on the analysis of samples harvested at the end of the stationary growth period for each culture condition tested. Results are directly comparable to those found for the analysis of samples from the end of the exponential growth period. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for each condition tested +/- STD.

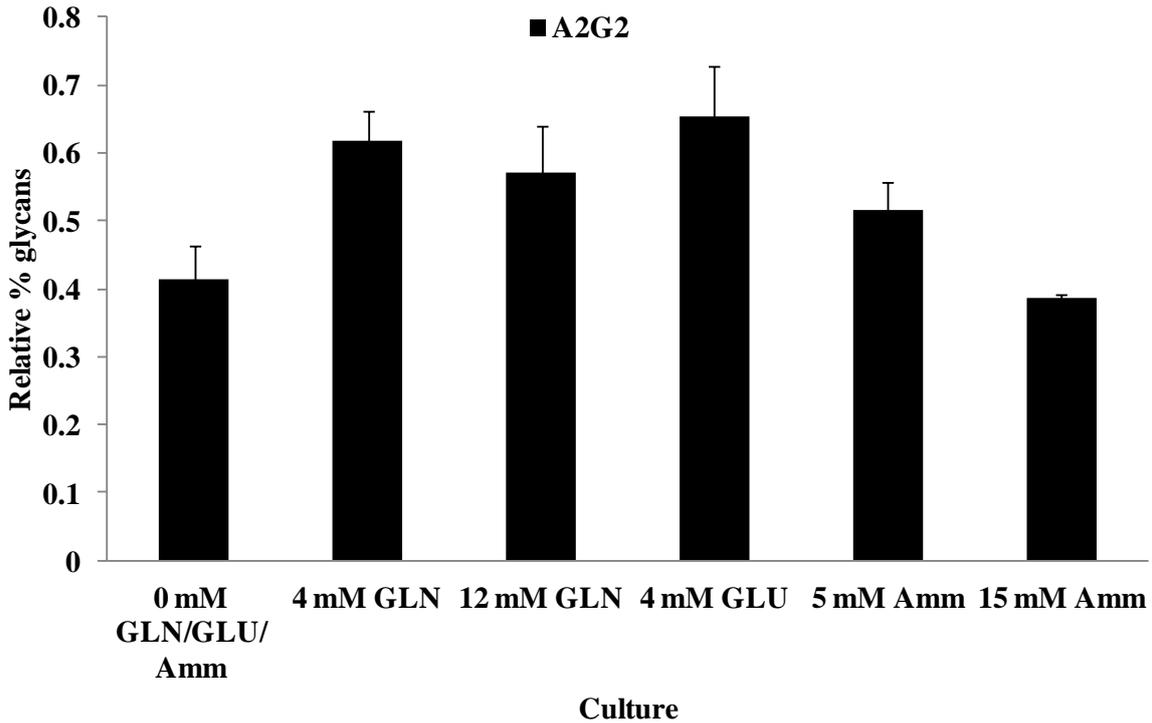


Figure 3.2.4.7: Relative % of A2G2 glycan forms detected on rIgG1 samples harvested from cultures containing varying concentration of L-GLN, L-GLU and ammonia. A2G2 was detected using HILIC following PNGaseF release and 2-AB labelling. The results above are based on the analysis of samples harvested at the end of the stationary growth period for each culture condition tested. Results are directly comparable results to those found for the analysis of samples from the end of the exponential growth period. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for each condition tested +/- STD.

Complex glycan processing may also result in the addition of a sialic acid residue onto one of the galactose residues present on F(6)A2G2 resulting in a glycoform termed F(6)A2G2S1. Figure: 3.2.4.8 shows the relative percentage of F(6)A2G2S1 present on rIgG1 purified from each of the cultures at the end of the exponential phase of growth.. The highest relative % of F(6)A2G2S1 sialyated glycoforms was present in the cultures containing 4 mM and 12 mM L-GLN. The cultures contained 71 % and 64 % higher relative % of the glycoform respectively in comparison to the 0 mM culture. GLN therefore appears to be essential for complex glycan processing, in particular in relation to sialylation. The 5 mM ammonia and 15 mM ammonia cultures had a 44 % and 29 % higher level of F(6)A2G2S1 respectively than the 0 mM culture. It may be observed that complex glycan processing was completed

due the inclusion of 4 mM L-GLN in these cultures. However the level of sialylation was 48 % and 59.6 % lower respectively than that of the 4 mM L-GLN cultures indicating an effect of higher ammonia concentrations on the sialylation of glycans. The inclusion of 4 mM L-GLU in culture media did resulted in a 25 % increase in the level of sialylation in comparison to the 0 mM culture. However the level of sialylation in the 4 mM L-GLN culture was 62 % higher than in that observed in the 4 mM L-GLU culture, indicating the necessity for L-GLN for the sialylation of glycans.

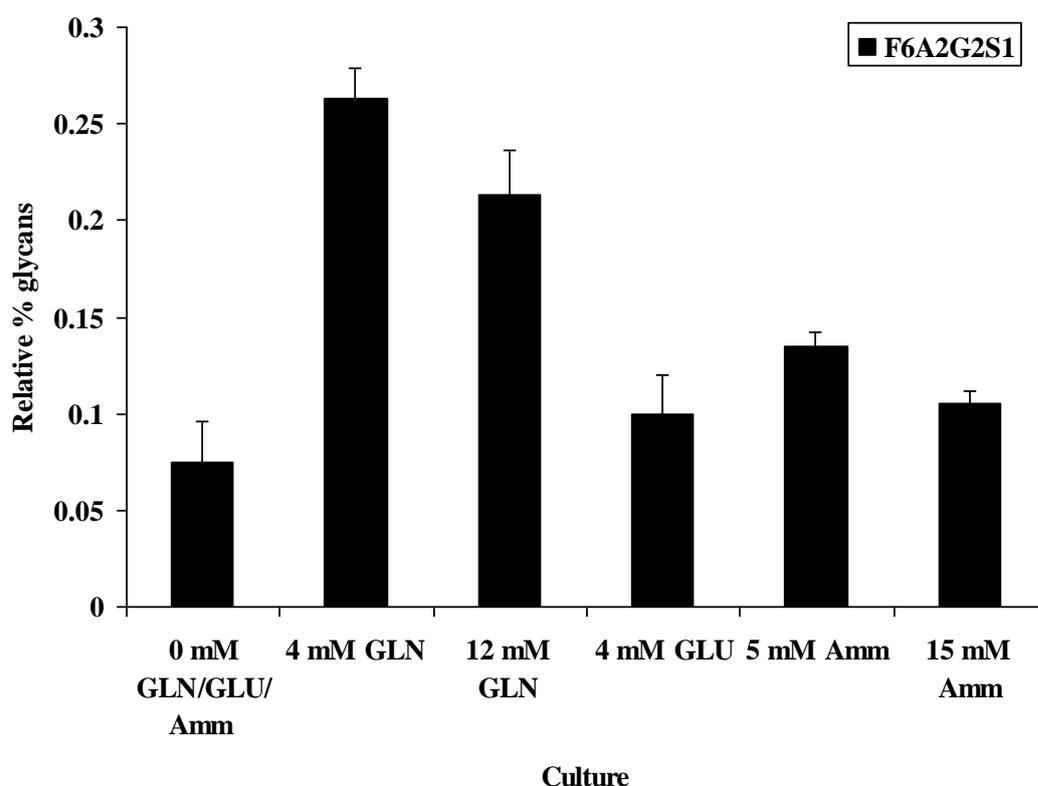


Figure 3.2.4.8: Relative % of F(6)A2G2S1 glycan forms detected on rIgG1 samples harvested from cultures containing varying concentration of L-GLN, L-GLU and ammonia. F(6)A2G2S1 was detected using HILIC following PNGaseF release and 2-AB labelling. The results above are based on the analysis of samples harvested at the end of the stationary growth period for each culture condition tested. Results are directly comparable to those found for the analysis of samples from the end of the exponential growth period. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for each condition tested +/- STD.

The impact that varying the concentration of L-GLN, L-GLU and Ammonia may have on the aggregation of the rIgG1 protein at the upstream level was investigated. SEC analysis was carried out on purified rIgG1 samples harvested from the cultures throughout the cultivation period. Figure 3.2.4.9 below represents the relative percentage of monomer present in the purified samples. As it may be noted, over 96 % of the protein harvested from all cultures was in a monomeric form. These results indicate no direct impact of varying the concentrations of L-GLN, L-GLU or ammonia on rIgG1 aggregation at the upstream level.

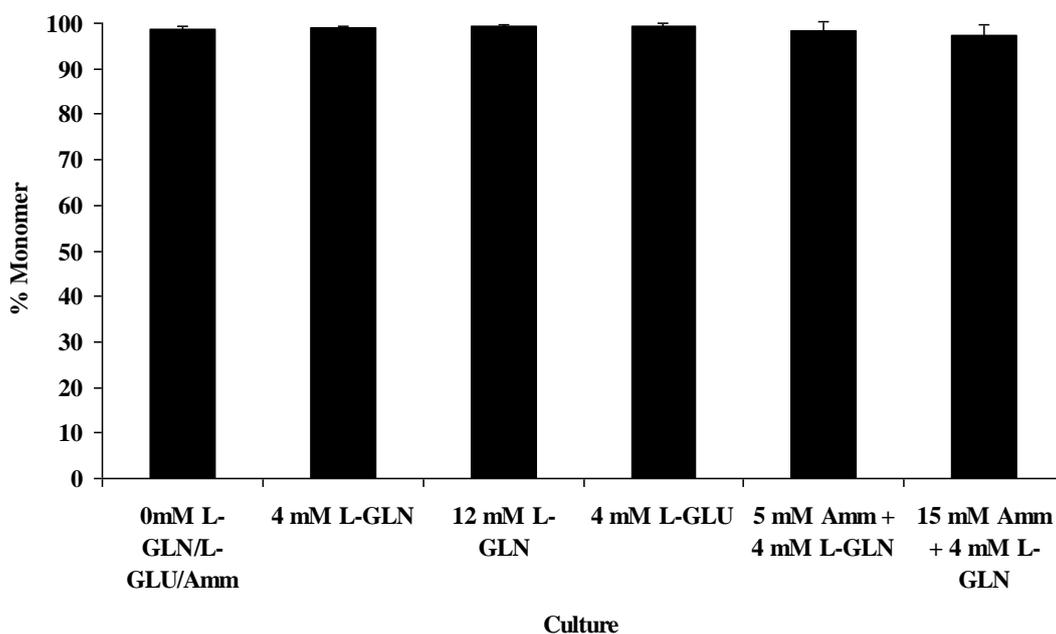


Figure 3.2.4.9: Relative % of monomeric form of rIgG1 samples as detected by SEC. IgG1 samples were harvested from cultures containing varying concentrations of L-GLN, L-GLU and ammonia. The above results are based on the analysis of samples harvested at the end of the stationary growth period. The results for samples harvested over the culture period in each of the cultures are directly comparable (data not shown). IgG1 samples were analysed in duplicate. The results are presented as the average of triplicate cultures performed for each of the conditions tested +/- STD.

3.3: Discussion:

Culture conditions were varied with regards to the initial concentration of GLN (0-12 mM), GLU (0-4 mM) and Ammonia (5-15 mM) present in the cultures. Such studies enabled the determination of the effect of increased L-GLN concentrations, L-GLN substitution and

high initial ammonia concentrations on cell growth, productivity and product quality. The underlying aim of the work was to investigate the critical ammonia level, above which cell growth, productivity and product quality may be adversely affected. Utilisation of L-GLN by cells in culture results in the accumulation of ammonia as a metabolic byproduct, with a maximum yield ratio of 1 mole of glutamine: 2 moles of ammonia. The incorporation of alternative carbon energy sources, such as L-GLU, in place of L-GLN is suspected to give rise to decreased ammonia accumulation as it is more chemically stable than L-GLN. Results to date investigating critical ammonia concentrations affecting product quality, in regards to glycosylation, involve the introduction of high NH_4Cl concentrations at the beginning of the culture. No comparisons are however made between the results obtained for high initial ammonia concentrations and conditions in which comparably high ammonia concentrations arise throughout the culture period. The results of the accumulation of a high concentration of ammonia throughout the culture period in the 12 mM L-GLN culture may be compared and contrasted to the results obtained from the 15 mM Ammonia culture in order to decipher if realistic ammonia accumulation throughout the culture period is a more effective indication of critical ammonia levels

3.3.1: Effect of L-GLN boosting

L-GLN is an amino acid which is utilised by cells that have high energy demands and synthesise large amounts of proteins and nucleic acids in favour of glucose as a carbon energy source. The energy obtained from L-GLN, in the form of ATP, is rapidly utilised by fast growing mammalian cells. Anabolic pathways for glutamine utilisation results in the production of building blocks for biomass and product synthesis. (Scheider et al., 1995; Vriezen et al., 1997). Therefore ignoring the potential adverse affects associated with ammonia accumulation on growth and productivity limitations of mammalian calls in culture, the effect of increased L-GLN concentration (0-12 mM) on cellular activities was investigated.

Cells were seeded at $0.3 * 10^6$ cells/ml in a total culture volume of 300 ml ExCell CHO 325 PF media supplemented with 0 mM, 4mM and 12 mM L-GLN. The highest maximum specific growth rate, μ_{max} , 0.0312 (h^{-1}), was displayed by cells cultured in media containing 4 mM L-GLN, 65 % higher than that observed in cultures containing no L-GLN. Increasing the concentration of L-GLN in the media to 12 mM resulted in a minimal decrease in the maximum specific growth rate displayed by the cells, however the biomass concentration

obtained (4.22×10^6 cells/ml) was similar to the 4 mM L-GLN (4.3×10^6 cells/ml). Cells cultured in the presence of 12 mM L-GLN showed an increased exponential phase of approximately 1 day in comparison to the 4 mM L-GLN culture. These results are comparable to those obtained by Vriezen et al., (1997) in which the effect of glutamine limitation in feed media on growth of two murine hybridoma cell lines was investigated. An excess of L-GLN in feed media did not result in an increased cell concentration in either cell line investigated. Yang and Butler, (2000) also investigated the effect of varying L-GLN concentrations (2-20 mM) on the growth and productivity of an EPO producing CHO cell line. Similarly to the results obtained for this study, the maximum specific growth rate was obtained in cultures containing 4 mM L-GLN. However an excess of L-GLN at a concentration of 20 mM resulted in a reduced specific growth rate and negatively impacted on biomass production which was reflected in a 50 % decreased yield in comparison to the 4 mM L-GLN culture. The above two studies also investigated the effect of very low L-GLN concentrations on mammalian cell growth. However in this report cells were cultured in the absence of L-GLN. Cells cultured in the absence of L-GLN displayed a significantly reduced growth rate, 65 % lower than that observed in the 4 mM L-GLN culture. Cells remained in a lag phase for 6 days before they entered exponential phase at the reduced maximum specific growth rate. The maximum viable cell density attained in such cultures was 51 % and 42 % lower than that achieved in the 4 mM and 12 mM L-GLN cultures respectively. Similarly Burleigh et al., (2011), reported lower viable cell densities in the first 3 days of the culture period for human chorionic gonadotropin (HCG) producing CHO cells grown in the absence of L-GLN in comparison to cells grown in concentrations of 4 and 8 mM L-GLN. However, growth was not limited to the same extent as was noted in this study, as the cells displayed a higher maximum specific growth rate at later stages of the culture in comparison to the cultures containing L-GLN. Rajendra et al., (2012) looked at the effect of L-GLN reduction of CHO and human embryo kidney (HEK)-293E cells and reported that the absence of L-GLN had a negative effect on cell density and cell viability. The results so far do indicate that L-GLN is necessary in culture media in order to promote cell growth. Limitation of cell growth in the absence of L-GLN is easily explained by the concept that L-GLN is a major carbon source which has metabolic fates giving rise to both energy production (catabolism) and building blocks for biosynthesis (anabolism). However, the lack of increased growth at higher L-GLN concentrations may be explained by investigating the cells metabolism.

L-GLN was completely consumed during the exponential phase of growth, at a rate of 0.89 mM/day, in cultures containing 4 mM L-GLN. The specific consumption rate displayed by the cells was 2.3×10^{-4} mmoles/cell/day. In 12 mM L-GLN cultures, all of the L-GLN was not consumed, even as cells entered death phase. The rate of L-GLN consumption during the exponential growth phase was higher than of the 4 mM L-GLN cultures, 1.34 mM/day. The cells cultured in the presence of 12 mM L-GLN displayed a specific L-GLN consumption rate of 3.8×10^{-4} mmoles/cell/day. Overall a trend for increasing L-GLN utilisation in the presence of high L-GLN concentrations was observed; similar to the results obtained for an EPO producing CHO cell line cultured in the presence of 2, 4 and 20 mM L-GLN (Yang and Butler, 2000). It may be concluded that the growth of cells in the 4 mM L-GLN culture became limited by the availability of GLN at the end of the exponential phase. L-GLN however was not limiting in the 12 mM L-GLN culture at the end of the exponential growth phase. It has previously been explained that the generation of ammonia in high L-GLN cultures is likely to be the inhibitory factor causing a reduction in growth rate. As the purpose of this study was to investigate L-GLN as a growth limiting substrate for this cell line and also the effect of accumulating ammonia on cellular activities, it is important to note that the concentration of ammonia was ~ 7 mM when the cells present in the 12 mM L-GLN culture ceased growing exponentially. Yang and Butler, (2000) observed that in the presence of 10 mM NH_4Cl , cell yield was reduced by 19 %. The specific ammonia production rate was 25 % and 21 % higher than glutamine in the 4 mM L-GLN and 12 mM L-GLN cultures. As the specific production rates of ammonia changed somewhat proportionally to the specific consumption rates of L-GLN in the cultures, no major changes in the pathways of glutamolysis occurred between the two different culture conditions, similar to results displayed by Yang and Butler, (2000). This is verified by the fact that yield of ammonia from glutamine is similar for both culture conditions tested, 1.07 mmol/mmol and 1.05 mmol/mmol for 4 mM and 12 mM L-GLN cultures respectively. Therefore it may be proposed that the higher ammonia concentration present in the 12 mM L-GLN culture, resulted in the transfer of cells from exponential growth phase into stationary growth phase. Such results may be cell line specific as no significant difference was observed in the accumulation of ammonia in cultures containing 4 mM and 8 mM L-GLN for a HCG producing CHO cell line (Burleigh et al., 2012). The concentration of ammonia present in the 0 mM L-GLN cultures remained below 2 mM for the entire exponential growth period. These results are comparable with those displayed by Wong et al., (2004). CHO cells cultured in the

presence of a feed concentration of 0.3 mM L-GLN displayed a reduced specific production of ammonia. However again, these results are cell line specific. For CHO cells producing HCG (Burleigh et al., 2012), a significant accumulation of ammonia was evident in the presence of 0 mM L-GLN. However, the growth kinetics displayed by such cells was also somewhat different to that which was observed in this current study.

As stated above L-GLN may be utilised by cells in favour of glucose as a carbon energy source, in cases of glucose limitation. In such cultures, glucose was not limited, but remained constant, as defined by the media used, at a concentration of ~17.4 mM. For both the 4 mM and 12 mM L-GLN culture, a general trend was observed in that all glucose present in the cultures was consumed by the end of the stationary growth phase. No significant difference was observed in the specific consumption rates of glucose between the 4 mM L-GLN culture, 8.3×10^{-4} mmoles/cell/day, and 12 mM L-GLN culture, 7.7×10^{-4} mmoles/cell/day. These results are comparable to those observed for other CHO cell lines in which it was demonstrated that glucose consumption was not affected by the initial amount of L-GLN, in a range of 0-6 mM, present in cultures (Rajendra et al., 2012). However the results may also be cell line specific. For a CHO cell line producing the recombinant protein EPO, a decrease in the specific glucose consumption rate was observed for cultures containing increasing concentrations of L-GLN (Yang and Butler, 2000). Vriezen et al., (1997) demonstrated that such variations do exist between different cell lines. For two murine hybridoma cell lines investigated, one displayed a decreased glucose consumption rate with increasing L-GLN concentrations, whereas the second cell line displayed no apparent effect. There was no significant difference in the specific lactate production rate, or the yield of lactate from glucose, 0.95 mmol/mmol and 1.04 mmol/mmol respectively, for 4 mM and 12 mM L-GLN. For the 0 mM L-GLN culture, glucose was consumed by the end of the exponential growth phase. No significant difference in the specific rate of glucose consumption was observed in the 0 mM L-GLN culture, however, the increased duration of the exponential growth period resulted in complete consumption of glucose present. The fact that the glucose consumption rate remained the same, suggests that the lower specific growth rate of the cells was in fact due to the absence of L-GLN as an energy source for biomass production. In the case in which L-GLN is absent, glucose becomes the growth limiting substrate. Although in this case the cells did demonstrate the ability to grow on glucose in the absence of L-GLN, it is evident that L-GLN remains the critical component for optimised growth, denoted by the 65 % higher growth rate observed in the 4 mM L-GLN culture. These results again prove to be cell line

specific as CHO producing HCG displayed lower glucose consumption rates in the absence of GLN in comparison to cultures containing 4 and 8 mM L-GLN (Burleigh et al., 2011). A 2-fold increase in the specific lactate production rate was observed for the 0 mM L-GLN/L-GLU/Ammonia culture. The results suggest a less favourable utilisation of glucose, via the glycolytic pathway, by the cells resulting in increased lactate production. The highest yield of lactate from glucose was observed in the 0 mM L-GLN culture, 1.39 mmol/mmol.

The results of Protein A purification of rIgG1 from culture media harvested from day 4 onwards, do indicate significant differences in the relative productivities displayed by cells cultured in the presence of L-GLN. A 50 % higher concentration of rIgG1 was obtained at the end of the stationary growth phase in cultures containing 4 mM L-GLN. This is reflected by the enhanced specific production rate of 50 % observed in this culture in comparison to the 0 mM L-GLN culture. No significant difference in the specific productivity of cells cultured in the 4 mM and 12 mM L-GLN was observed. The results observed are similar to those documented by Burleigh et al., (2011). The concentration of HCG produced by CHO cells in 0 mM L-GLN was statistically lower than that produced in 4 mM L-GLN on day 5 of the culture period. However the effect of L-GLN concentration on recombinant protein production in mammalian cell lines does vary. For CHO cells producing the recombinant protein EPO, the concentration of EPO produced decreased with increasing L-GLN concentration from 2-20 mM. Cell specific productivities observed were reported highest in the 20 mM L-GLN culture (Yang and Butler, 2000). In CHO cells cultivated in 0-6 mM L-GLN, the highest rIgG concentration was observed in the 2 mM L-GLN culture. It was concluded that the overall improvement in IgG production at reduced GLN concentrations was due to the reduced ammonia accumulation observed (Rajendra et al., 2012). However in the case of this current study, rIgG1 production was not inhibited by the higher accumulation of ammonia in the 12 mM L-GLN culture, but by the absence of L-GLN in the 0 mM L-GLN culture.

The results so far indicate the necessity for L-GLN for optimised growth and recombinant protein production. It is also necessary to investigate if such growth and productivity patterns have an influence on the quality of the protein being produced. Higher cellular growth rates and production rates are routinely associated with lower quality recombinant proteins due to the limited time available for complex glycan processing. On the other hand, the lower growth and production rates in the absence of L-GLN may not in turn result in higher glycan processing due to the necessity of GLN for the synthesis of glycosylation nucleotide sugars

and their corresponding donors. Therefore increased L-GLN concentrations may in turn result in higher levels of glycan processing due to increased levels in the pools of nucleotide sugars and their donors.

In order to determine the influence of L-GLN and L-GLN concentration on rIgG1 quality, glycan analysis was completed on rIgG1 samples harvested from the cultures at the end of the exponential and stationary phases of growth. Irrelevant of the concentration of L-GLN present, all cultures displayed a heterogeneously similar trend in the range of glycan structures released from the rIgG1 samples. The highest relative percentage present was of the early, unprocessed glycoform such as M5 and F2A2. The lowest relative % were of the sialyated glycans.

The earliest form of glycosylation arising from the action of the oligosaccharyltransferase (OST) enzymes, followed by glucosidase and mannosidase enzymes present in the endoplasmic reticulum (ER) is M5. It is a high mannose structure consisting of the 2 N-acetylglucosamine (GlcNAc) residues and 5 mannose residues ($\text{Man}_5\text{GlcNAc}_2$) which are attached via a glycosyltic bond to the asparagine residue at position Asn297 at the hinge region of IgG1. This intermediate glycan structure typically undergoes more complex glycan processing in the golgi by a series of in house N-acetylglucosamine transferases, galactosyl transferases and sialy transferases enzymes. The highest relative percentage of this intermediate unprocessed glycan structure occurred in the 0 mM L-GLN culture. A 65 % and 60 % decrease in the relative percentage of M5 present in the 4 mM and 12 mM cultures was observed respectively. The lower relative % of M5 in these cultures was in fact due to the presence of a higher relative % of fully fucosylated, galactosylated and sialyated F6A2G2S1 glycans. There was no significant difference observed in the relative % of glycans containing one galactose residue at the α 2,3 or α 2,6 positions or glycans containing two galactose residues. Similar results were noted for the glycosylation of HCG produced in CHO cell cultures containing 0, 4 and 8 mM L-GLN. At the end of the culture period, a decrease in sialylation, fucosylation and antennarity was noted in cultures containing 0 mM (Burleigh et al., 2011). Wong et al., (2004) observed that glycan chain extension and sialylation may be affected by glutamine limitation. In fed-batch cultures of CHO cells producing the recombinant protein IFN- γ , decreased sialylation and increased high mannose structures were reported for an L-GLN feed concentration of 0.1 mM in comparison to that of 0.3 mM. A 23 % decrease in sialylation was observed for 0.1 mM L-GLN fed-batch cultures, in comparison to 4 mM L-GLN batch cultures.

The mechanism by which L-GLN limitation effects complex glycan processing is explained by an associated intracellular decrease in the availability of the nucleotide sugar donor UDP-GlcNAc, brought about by a lack of synthesis the amino sugar N-acetylglucosamine 1-P (Nyberg et al., 1999). Complex processing in the golgi initiates with the cleavage of $\text{Man}_5\text{GlcNAc}_2$, to form $\text{Man}_3\text{GlcNAc}_2$ by the action of the enzyme Mannosidase II. A series of N-acetylglucosamine transferase (GnT) enzymes are responsible for the addition of GlcNAc residue from the nucleotide sugar donor UDP-GlcNAc onto the free $\alpha 1,3$ and $\alpha 1,6$ mannose arms of $\text{Man}_3\text{GlcNAc}_2$, thus forming a biantennary structure. This biantennary structure is further processed by a series of galactosyl and sialy transferase enzymes. As seen in Figure 3.3.1.1, below, a reaction catalysed by glutamine:fructose-6-P amidotransferase (GFAT), sees the transfer of ammonia from glutamine to fructose-6-phosphate, forming glucosamine-6-phosphate. This step serves as the limiting step in the determination of the formation of the amino sugar. Therefore under conditions of glutamine limitation, a decrease in the pool of UDP-GlcNAc may be observed, typically resulting in a decreased level of complex glycans and an increased level of intermediate glycan structures such as M5 (Nyberg et al., 1999). However in the case of this study, complex glycan processing in the form of biantennary structures and galactosylation did occur in the same extent in the 0 mM L-GLN culture, as was observed in the 4 and 12 mM L-GLN, indicating that the pool of UDP-GlcNAc residues may have been unaffected by the lack of GLN. The increased ratio of M5 in the 0 mM L-GLN culture coincides with a decreased ratio of glycan structures containing sialic acid residues. L-GLN limitation is known to affect sialylation, again through altering the intracellular pool of UDP-GlcNAc nucleotide sugar donors. UDP-GlcNAc is a necessary precursor for the synthesis of the sialic acid donor CMP-NANA (Burleigh et al., 2011). A process consisting of 5 enzymatic steps resulting in the fusion of a hexose and a 3 carbon pyruvate structure cumulates in the conversion of UDP-GlcNAc into CMP-NANA (Butler, 2006). It was stated above that the intracellular pool of UDP-GlcNAc does not appear to have been altered in the 0 mM L-GLN culture as the proportion of biantennary and galactosylated structures remained consistent with the 4 and 12 mM, L-GLN cultures. However the lack of sialylation observed in the 0 mM L-GLN culture suggests that there was a decrease in the pool of UDP-GlcNAc under the glutamine limitation conditions induced. This decreased pool did not alter the level of biantennary structures, but did influence the availability of CMP-NANA as a sialic acid precursor. It has been suggested that as sialylation is the last step in N-glycan processing, it is probably more sensitive to the effects of substrate

depletion in comparison to the other steps of glycan processing (Wong et al., 2004). It is also important to note that the inclusion of a higher concentration of L-GLN (12 mM) did not significantly enhance the degree of complex glycan processing in comparison to the 4 mM L-GLN culture.

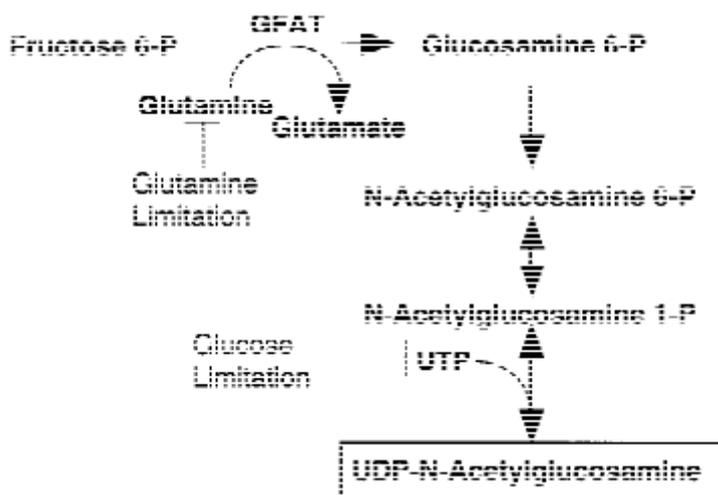


Figure 3.3.1.1: Role of glutamine and glucose in nucleotide sugar donor synthesis Nyberg et al., (1999)

3.3.2: Substitution of L-GLN with L-GLU

A study was designed in order to determine if L-GLN replacement with L-GLU would result in any significant difference in the growth, metabolism, productivity and quality of the product produced in the CHO DP-12 cell line. There are a number of possible outcomes associated with the substitution of L-GLN with L-GLU as an alternative carbon source. As L-GLU has only one amino group in its structure, in comparison to the two present in L-GLN, and is transported into the cell at a lower velocity, more efficient cell metabolism should result, thus leading to reduced ammonia formation. However as noted above in the introduction, the amino groups of L-GLN and L-GLU have both catabolic and anabolic fates required for both energy and biomass building block production. Therefore the replacement of L-GLN, containing 2 amine groups, with a carbon source containing one amine group, may result in reduced energy production, giving rise to decreased cellular productivity. Also as noted in Section 3.3.1, the ammonia of L-GLN plays an essential role in maintaining the levels of nucleotide sugar donors, UDP-GlcNAc, at sufficient levels to allow

for complex glycan processing. In order to evaluate the effects of L-GLN replacement with L-GLU, cells were seeded at 0.3×10^6 cells/ml in a culture volume of 300 ml ExCell CHO 325 PF media supplemented with either 4 mM L-GLN, 4 mM L-GLU or 0 mM L-GLN/L-GLU.

The maximum viable cell density attained in the 4 mM L-GLU culture was 46 % lower than that in the 4 mM L-GLN culture. Cell cultured in the presence of L-GLU grew at a reduced maximum specific growth rate, 0.0096 h^{-1} , in comparison to that displayed by cells culture in L-GLN, 0.0312 h^{-1} . There was no significant difference in the maximum specific growth rate or maximum viable cell density achieved between cells culture in 0 mM L-GLN/L-GLU and cells cultured in 4 mM L-GLU. These cultures did however show increased culture longevity with viability remaining above 86 % until day 16 for the 0 mM L-GLN/L-GLU culture and until day 11 for the 4 mM L-GLU culture. Viability was only maintained at such a level until day 7 in the 4 mM L-GLN culture. These results are comparable with those obtained by Hong et al., (2010) for CHO cells producing rIgG1. A lower growth rate and maximum viable cell density and increased culture longevity was noted for cells cultured in 6 mM L-GLU in comparison to those cultured in the presence of 6 mM L-GLN. These results are however noted to be cell line specific. In other studies, the substitution of L-GLN with L-GLU in a glucose based media resulted in a higher final cell concentration (Altamirano et al., 2000). The cell line used for this study, conducted by Altamirano et al., (2000), displayed glutamine synthase (GS) activity in which L-GLN could be formed from L-GLU. The replacement of L-GLN with L-GLU has also been associated with enhanced rIgG1 production (Hong et al., 2010). However these results may be cell line specific, as the replacement of L-GLN with L-GLU did not affect the specific production rate displayed by the cells. A decrease of 40 % and 21 % in the concentration of rIgG1 present at the end of the exponential and stationary phase respectively was present in the 4 mM L-GLN culture in comparison to the 4 mM L-GLU culture. This increased rIgG1 concentration present, was however due to an extended culture period in the 4 mM L-GLU culture.

The substitution of L-GLN with L-GLU did not appear to be beneficial in regards to biomass production. However one associated positive aspect of the substitution lies in the reduced accumulation of ammonia in L-GLU cultures. Throughout the exponential growth period, the concentration of ammonia in the 4 mM L-GLU remained below 1.3 mM, whereas a concentration of 4.9 mM ammonia was present in the 4mM L-GLN culture at the end of the exponential growth period. Such results are comparable to those observed by Hong et al.,

(2010), in that the accumulation of ammonia was said to be 3-fold lower in glutamate cultures than that observed in glutamine cultures. However the beneficial effects of reduced ammonia concentration appear to be outweighed by the negative impact replacement of L-GLN with L-GLU has on cell growth. Glucose metabolism did appear to be affected by the substitution of L-GLN with L-GLU. The specific glucose consumption rate displayed by the cells cultured in 4 mM L-GLU was 40 % and 33 % lower than that observed in the 0 mM L-GLN/L-GLU and 4 mM L-GLN cultures. This fact highlights the relationship between glycolysis and glutaminolysis and their interconnection. The substitution of L-GLN with L-GLU has been associated with a decrease in the rate of glutaminolysis. The reduction in this rate results in a reduced requirement for pyruvate to be incorporated into the tricarboxylic acid (TCA) cycle, and thus the glycolysis rate may be reduced (Altamirano et al., 2000). It is often reported that decreased glucose consumption rates is coupled with decreased lactate production rates in situations of L-GLN substitution (Hong et al., 2010; Altamirano et al., 2000). Such results were not observed as the specific lactate production rate was not significantly different to that observed in 0 mM L-GLN/L-GLU cultures, with a similar lactate yield from glucose of 1.35 mmol/mmol being determined for the 4 mM L-GLU culture. Therefore the combination of reduced glucose consumption rates, coupled with increased lactate production rates would suggest a similar inefficient metabolism of glucose by cells cultured in the presence of L-GLU in comparison to 0 mM L-GLN/L-GLU/Amm.

The substitution of L-GLN with L-GLU also had negative effects in regards to the quality of the glycan structure attached. The results obtained are directly comparable to the 0 mM L-GLN/L-GLU culture in that a higher relative % of M5 were detected while a lower relative % of sialyated structures were detected, in comparison to the 4 mM L-GLN culture. Other studies report on the positive influence L-GLU has on galactosylation in comparison to glutamine (Hong et al., 2010). The formation of galactosylated, biantennary structures was not influenced by the substitution of L-GLN with L-GLU. The reasoning for such a display of glycans may also be explained by a decrease in the intracellular concentration of UDP-GlcNAc, as above. It may be proposed that the incorporation of L-GLU into the culture did result in the formation of a sufficient quantity of UDP-GlcNAc to allow for biantennary fucosylated glycan processing. However, the supply of UDP-GlcNAc available for CMP-NANA formation may have been limited in comparison to cultures containing 4 mM L-GLN.

3.3.3: Influence of high initial ammonia concentrations

Many studies to date investigating the effects of ammonia accumulation on not only growth and productivity of mammalian cells, but also product quality, have focussed on the inclusion of high initial concentrations of ammonia in the form of NH_4Cl (0-30 mM) (Borys et al., 1994; Anderson and Goochee, 1995; Gawlitzek et al., 2000; Yang and Butler, 2000; Yang and Butler, 2002) Other studies have looked at increasing ammonia concentration throughout the culture period by the inclusion of varying concentrations of NH_4Cl feed media (Hansen and Emborg, 1994; Gawlitzek et al., 1998).

The concentrations of ammonia added may have been typical of the reported accumulations of ammonia in standard mammalian cell cultures (Anderson and Goochee, 1995). However many studies failed to investigate the significance of the difference between increased ammonia accumulation arising throughout the culture period, as a result of cell metabolism and/or chemical degradation or high concentrations of ammonia added exogenously, in the form of NH_4Cl , at some stage during the culture period. Gawlitzek et al., (2000) attempted to investigate this by conducting studies with CHO cells producing TNFR-IgG in which initial concentrations of L-GLN were varied between 1-11 mM and ammonium between 0-13 mM. However in this study cellular growth was arrested before the cells were subjected to such concentrations. Therefore in order to investigate the effect of both high initial ammonia concentrations (typical of that which arises during a standard mammalian cell culture) and the effect of ammonia which arises due to cell metabolism, cells were cultured in the presence of an initial concentration of 5 and 15 mM NH_4Cl . The inclusion of 5 mM ammonia at the beginning of the culture period is comparable to the culture which contained 4 mM L-GLN. 4 mM L-GLN gave rise to an accumulation of 5 mM Ammonia. The inclusion of an initial concentration of 15 mM ammonia is comparable to the 12 mM L-glutamine culture, as this culture gave rise to the high ammonia concentration of 13 mM.

The growth rate of the cells appeared to be effected by ammonia in a dose-dependent manner. Cells cultured in the presence of 15 mM ammonia had a 33 % lower maximum specific growth rate, than that displayed by cells cultured in the presence of 5 mM ammonia. The maximum viable cell density achieved in the 15 mM ammonia culture was 31 % lower than that attained in the 5 mM ammonia culture. These results are similar to those presented by Yang and Butler, (2000), in which the biomass yield for an EPO producing CHO cell line decreased with increasing initial concentrations of NH_4Cl ranging from 0-40 mM. In this study, it was reported that the inhibitory concentration of ammonia for a 50 % (IC-50)

decrease in biomass yield was 33 mM. Chen and Harcum, (2006) also reported a reduced growth rate in the presence of high initial ammonia concentrations of 10 mM, in comparison to a standard culture containing 7 mM L-GLN (concentration of ammonia in standard culture did not reach above 4 mM). In this current study, the maximum viable cell density occurred in the 4 mM L-GLN culture. The maximum viable cell density achieved in the 15 mM ammonia culture was 59 % lower than that achieved in the 4 mM L-GLN culture, indicating that 15 mM ammonia is the approximate IC-50 values for this cell line. The influence of an initial ammonia concentration of 5 mM in comparison to the influence of an accumulation of 5 mM ammonia (as seen in 4 mM L-GLN culture) resulted in a 30 % decrease in the maximum specific growth rate displayed by the cells. The incorporation of a high initial ammonia concentrations (15 mM), also resulted in a 54 % decrease in the maximum specific growth rate of the cells when compared to the 12 mM L-GLN culture (gave rise to accumulation of a total of 13 mM Ammonia on day 6). Therefore, the effect of high initial ammonia concentrations on cell growth may be more profound than the actual implications of high ammonia accumulations throughout the culture period. It was stated in the Section 3.3.1 that cells cultured in the presence of 12 mM L-GLN left the exponential growth phase when L-GLN was still available for consumption. The concentration of ammonia present in the culture at this point was ~ 7 mM and it was hypothesised that this concentration of ammonia may have cause a cease in the exponential growth phase. However, cells cultured in initial ammonia concentrations ranging between 5-15 mM did exhibit an exponential growth period and thus growth was not inhibited but reduced between these concentrations. Therefore it may also be proposed that the cessation of growth in the 12 mM L-GLN culture may be due to the depletion of some other media component. Cells cultured in the presence of 5 and 15 mM ammonia also entered stationary phase before L-GLN was depleted. Therefore it may be deduced that in the presence of higher concentrations of ammonia, other media components become the growth limiting substrate.

The specific L-GLN consumption rate displayed by the cells was similar in both the 5 mM and 15 mM ammonia culture. The specific L-GLN consumption rate displayed by the cells cultured in an initial concentration of 5 mM ammonia was 52 % higher than that displayed in cultures containing 4 mM L-GLN. Similarly the specific L-GLN consumption rate displayed by the cells cultured in the presence of an initial concentration of 15 mM ammonia was 22 % higher than that of the cells cultured in 12 mM L-GLN. Similar results were noted for in studies investigating the effects of a range of high ammonia concentrations on cellular

activities. Hansen and Emborg, (1994) found that L-GLN was extensively consumed by not completely depleted when ammonia concentrations ranged between 0-7.5 mM. In this current study the inclusion of high initial concentrations of ammonia resulted in a change in metabolism illustrated by an increased specific L-GLN consumption rate coupled with reduced ammonia accumulation. Similar results were reported by Hansen and Emborg, (1994) for CHO cells producing tissue plasminogen activator (t-PA). Feed media containing 5 and 7.5 mM ammonia resulted in a final culture ammonia concentration 1 and 0.5 mM respectively, even though L-GLN was extensively consumed in these cultures. In this current study the yield of ammonia from L-GLN consumed was significantly lower in the 5 mM and 15 mM ammonia cultures, 0.64 and 0.63 mmol/mmol respectively, in comparison to that which was determined for the 4 mM and 12 mM L-GLN cultures, 1.07 and 1.05 mmol/mmol respectively. Cells have a mechanism to ensure that less ammonia is generated during growth under high ammonium concentrations. This shift in metabolism was explained by a shift from the glutamate dehydrogenase pathway to the use of the alanine amidotransferase pathway. This shift is initiated by the conversion of L-GLU to 2-oxoglutarate. Hansen and Emborg, (1994) reported an increase of alanine by 30 % under high ammonia concentrations. Therefore the inclusion of high initial ammonia concentrations does suggest an alternative effect on cellular metabolism than that which is observed when high ammonia concentrations accumulate in culture. However, the results may be cell line dependent. Yang and Butler, (2000) illustrated that for CHO producing EPO, high L-GLN consumption rates are coupled with high ammonia production rates under high concentrations of ammonia.

The specific glucose consumption rate displayed by cells cultured in 15 mM Ammonia was 30 % higher than that of the 5 mM Ammonia culture and also the 4 and 12 mM L-GLN cultures. Therefore only very high initial ammonia concentrations effect glucose metabolism. These results are different to those reported by Hansen and Emborg, (1994) which report a decrease in the specific glucose consumption rate with increasing concentrations of ammonia. However for a CHO cell line producing the recombinant protein EPO, the specific glucose consumption rate was noted to increase with increasing ammonia concentrations from 0-40 mM (Yang and Butler, 2000). Cells cultured in the presence of 15 mM ammonia had a higher specific lactate production rate than that displayed by cells cultured in the presence of 5 mM ammonia. However this did not result in an increased accumulation lactate due to the lower cell numbers present in the culture. As both the glucose and lactate specific production rates were noted to increase in the 15 mM ammonia culture, no significant difference in the yield

of lactate from glucose would be expected, which was determined. The yield of lactate from glucose was similar in the 15 mM ammonia culture, determined as 0.92 mmol/mmol, to those observed in the 5 mM Ammonia, 0.98 mmol/mmol, and 4 mM and 12 mM L-GLN cultures, 0.95 and 1.04 mmol/mmol respectively. The increased lactate production rate indicates inefficient glucose metabolism displayed by cells cultured in the presence of high initial ammonia concentrations. Glucose metabolism is favoured towards glycolytic and opposed to oxidative for ATP production (Hansen and Emborg, 1994). Such inefficient glucose metabolism is not noted in the presence of 5 mM ammonia or in situations in which high ammonia concentrations accumulate in the cultures.

Typically cells cultured in the presence of increasing initial concentrations of ammonia display decreased specific recombinant protein productivities attributed to possible losses of productivity or adverse depletion of metabolites necessary for cell production under high ammonia concentrations (Hansen and Emborg, 1994). However the effect of high initial ammonia concentrations may be cell line specific. For CHO cells producing HGC no difference was displayed in the secretion rate at ammonia concentrations ranging from 0-25 mM (Anderson and Goochee, 1995). For CHO cells producing TNFR-IgG, Gawlitzek et al., (2000) also reported no difference in productivity at varying initial ammonia concentrations ranging from 0-13 mM. However for CHO cells producing the recombinant protein EPO, increased specific productivity was noted at initial ammonia concentrations of 40 mM in a study which investigated the effects of initial ammonia concentrations ranging from 0-40 mM (Yang and Butler, 2000). It should also be noted that for CHO cells producing rMouse placental lactogen-I, specific production rate was noted to decrease with increasing ammonia concentrations over the range 0-9 mM. Production was inhibited at 9 mM ammonia (Borys et al., 1993). In this current study there was no significant difference in the specific rIgG1 production rate demonstrated between cells cultured in 5 and 15 mM ammonia. Cells cultured in the presence of 15 mM ammonia displayed a 26 % increase in the specific rIgG1 production rate in comparison to cells cultured in the presence of 12 mM L-GLN. However due to the adverse effects of high initial ammonia concentrations on cell growth, no direct increase in the quantity of rIgG1 produced at the end of the exponential or stationary phase was observed in this culture.

Variations were noted between the levels of the different glycoforms present on samples taken from the cultures containing the two different initial ammonia concentrations. There was a 45 % increase in the proportion of the intermediate glycan M5 in samples harvested

from the 15 mM ammonia culture in comparison to that observed on samples taken from the 5 mM ammonia culture. Due to the increased levels of the intermediate unprocessed glycan in the 15 mM ammonia culture, an expected decrease in the relative proportions of processed galactosylated and sialylated glycans was noted between the 5 and 15 mM ammonia cultures. There was a 22 % and 26 % decrease in the proportions of glycans containing one galactose residue, F6A2[6]G(4)1 and F6A2[3]G(4)1 respectively, in the 15 mM ammonia culture in comparison to the 5 mM ammonia culture. Fully galactosylated glycans were reduced by 29 % in the higher ammonia concentration culture. There was a 22 % reduction in the relative % of the sialylated glycan F(6)A2G2S1, when the initial concentration of ammonia increased from 5 to 15 mM. Borys et al., (1993) demonstrated that inhibition of mouse placental lactogen-I glycosylation correlated with the concentration of ammonia species with adverse effects being noted as the concentration of ammonia is increased from 0-9 mM.

In literature the effects of increasing initial ammonia concentrations have been associated with increased proportions of bi-antennary glycan such as F6A2, coupled with decreased glycan processing in regards to sialylation. Anderson and Goochee, (1995) reported for CHO producing granulocyte colony-stimulating factor an initial ammonia concentration of 2 mM was noted to significantly reduce O-linked glycosylation. A decrease in the disialylated forms relative to monosialylated forms was noted at an ammonia concentration of 50 mM. Gawlitzek et al., 1998 illustrated that for BHK-21 cells producing human interleukin-2 (IL-2), a 45 % increase in high branched complex structures was noted in cultures containing 15 mM ammonia in comparison to standard culture conditions in which ammonia accumulation was reported between 2 and 4 mM. Zanghi et al., (1998a&b) reported that 10 mM ammonia gave rise to a 10 % decrease in the polysialic content of neural adhesion molecule (NCAM) expressed in CHO cells. Gawlitzek et al., (2000) noted that the galactose and sialic acid content of TNFR-IgG expressed in CHO cells decreased with increasing medium ammonia concentrations in the range of 0-13 mM. However in this situation no differences in branching were observed. Yang and Butler, (2000) noted that the relative proportion of EPO with 4 sialic acid residues was reduced with increased ammonia concentrations in the range of 30 mM. However a decrease in antennary was noted with a 57 % reduction in the proportion of tetraantennary structures. There have been limited reports on the effect of increasing ammonia concentrations on the galactosylation profile of glycan structures. Hong et al., (2010) reported an increase in ungalactosylated structures, coupled with a decrease in

structures containing one or two galactose residues as the concentration of ammonia increased from 0-15 mM.

The results to date therefore appear to be consistent in that the galactosylation and sialylation of glycoproteins is adversely affected by increasing initial concentrations of ammonia. Such results were observed in this current study. However the impact of high ammonia concentrations on glycan antennary appears to be cell type or line specific. In studies in which more complex glycosylation has been noted for increased initial ammonia concentrations it was noted observed that the increased complexity was directly related to an increase in the intracellular content of UDP-GNac which increase the availability for GlcNAc and GalNAc residues to be attached to the emerging polypeptide chain (Gawlitzeck et al., 1998). As it was explained in Section 3.3.1, the formation of UDP-GNac requires the donation of ammonia to fructose-6-phosphate for conversion to glucosamine-6-phosphate. Therefore increased ammonia levels may in turn increase the formation of nucleotide sugar donors. Studies have also been conducted which note an increase in the activity of the enzyme glutamine:fructose-6-P amidotransferase in high ammonia concentrations of 15 mM (Calyli et al.,1999), which again promotes the formation of UDP-GNac. It was also reported by Gawlitzeck et al., (1998) that the corresponding increases in the pool of UDP-GNac's was noted in four different cell lines (BHK, CHO, Ltk929 and hybridoma) when subjected to high initial ammonia concentrations. It was later determined that the increased pool of UDP-GNac induced an increased level of activity in GnT (Grammatikos et al., 1998). Other studies have also reported such findings of increased intracellular pools of UDP-GNac in the presence of ammonia (Gawlitzeck et al., 1998 and Grammatikos et al., 1998). The results presented in this current study do not conform to this theory in that similar levels of bi-antennary structures were noted in cultures containing 5 and 15 mM ammonia. Also there was a decrease in the proportion of galactosylated glycans in the 15 mM ammonia culture in comparison to the 5 mM ammonia culture.

The theory behind decreased sialylation in the presence of high ammonia concentrations has been extensively studied and a number of mechanisms have been proposed. One such mechanism is based on the presence of a higher concentration of UDP-GNac pools in the cytoplasm, which are thought to impair the transport of CMP-NANA into the golgi, thus resulting in decreased sialylation (Pel Rijken et al., 1995). In Section 3.3.1 above, it was proposed that increased pools of UDP-GNac promote the formation of CMP-NANA thus leading to increased sialylation in the presence of L-GLN in comparison to situations in which

L-GLN is absent. However it is also proposed that the transport of CMP-NANA into the golgi apparatus may also be inhibited by increased concentrations of UDP-GNac. A feedback mechanism does exist in the pathway that forms CMP-NANA from UDP-GNac. Enzymes involved in this process are regulated by CMP-NANA concentration in the cytoplasm. High concentrations of CMP-NANA in the cytoplasm brought about by high concentration of UDP-GlcNAc are prevented from being transported into the golgi (Butler, 2006). However as it is unlikely in this study that higher concentrations of UDP-GNac did exist in the presence of increase ammonia concentrations, it is more probable that the decreased sialylation was due to alterations in the golgi pH brought about by the ammonia ions (Borys et al., 1994).

The results of decreased galactosylation and sialylation noted in this current study can perhaps be better explained by evaluating the effect of ammonia on glycosylation at the gene level. Lower expression levels for the galactosylation and sialylation enzymes, β 1,4-galctosyltransferase and α 2,3-sialytransferase, was observed under increased ammonium concentration conditions of 10 mM. Gene expression of CMP-SiaT was also sensitive to ammonium chloride, which would also reduce the level of sialylation. However this was only the case observed at early culture times. Previous studies reporting on decrease galactosylation suggested a decrease in β 1,4-galctosyltransferase activity due to an increase in the intracellular pH, brought upon by high ammonia concentrations (Gawlitzek et al.,2000). This statement correlates with the decreased gene expression noted by Chen and Hercum, (2006).

The impact of high initial ammonia concentrations on the glycosylation profile of the recombinant protein produced was notably different to that observed in cultures in which similar levels of ammonia accumulated over time throughout the culture period. A 98 % decrease in the proportion of glycan of M5 nature was noted in the 5 mM ammonia culture, in comparison to the culture which gave rise to an accumulation of 5 mM ammonia throughout the culture period. Similarly the effect of high initial ammonia concentrations in comparison to high ammonia accumulations resulted in a 150 % in the proportion of M5 glycans in comparison to a culture which gave rise to an ammonia accumulation of 13 mM. Corresponding decreases were noted in complex glycan structures when the 5 mM and 15 mM ammonia cultures were compared to the 4 and 12 mM L-GLN cultures, respectively. For example approximately a 50 % decrease in the proportion of sialyated glycans, F6A2G2S1, was noted between the 5 mM ammonia and 4 mM L-GLN culture and 15 mM ammonia and

12 mM L-GLN culture. The inclusion of high initial ammonia concentrations has enabled the understanding of the mechanisms by which high ammonia concentrations (typical of the expected concentration accumulated over a culture period) affect cell growth and productivity. However this work has established that the results due to some extent over-estimated the impact of an accumulation of a high concentration of ammonia in a mammalian cell culture. In order to verify that alterations in cell growth, metabolism, productivity and product quality obtained for the 15 mM ammonia culture were not due to the adverse effects of an increased osmolality in the medium brought about through the addition of ammonium chloride, a similar study was repeated in which an initial concentration of 15 mM sodium chloride and 4 mM L-GLN were added. The results obtained for all cellular activities were not significantly different from the 4 mM L-GLN culture.

3.4: Conclusion

The main outcome of this work is that it has signified the importance of L-GLN in mammalian cell culture media for this particular CHO DP-12 cell line producing rIgG1. Optimised growth (growth rate and maximum viable cell density reached), productivity and product quality were achieved in cultures containing 4 mM L-GLN in comparison to cultures containing 0 mM L-GLN and 4 mM L-GLU. In conforming to the overall aim of the thesis, this study has highlighted the ideal concentration of L-GLN to incorporate into ExCell CHO 325 PF media for growth of the CHO DP-12 cell line. Increasing the L-GLN concentration to 12 mM did not improve cell or product yields or impact on product quality. The steps forward from this work will involve investigating if higher cell densities may be achieved, greater than the maximum observed in the 4 mM L-GLN culture in this study. An investigation for increased cell yields will be conducted through means of up-scaling of the suspension cultures to a 1.7 L minifors reactor platform and also encapsulation of the suspension cell line in alginate-poly-L-lysine-alginate microcapsules. Depending on the results achieved for higher cell density in the experiments to follow, various feeding strategies, such as continuous (perfusion), may be applied in order to further enhance any achievements in higher cell densities which are made. It is possible that if cells are cultured to higher cell densities than those achieved in this early study, the concentration of the growth limiting nutrient, L-GLN, may have to be increased in subsequent studies in order to meet the energy demands of the cells. Therefore this initial study in the thesis also investigated the effect of higher initial L-GLN concentrations (12 mM) on all cellular activities and

recombinant protein quality. The main aim of increasing the L-GLN concentration was not only to determine if it had a beneficial effect on cellular activities, but also to determine if the higher accumulation of ammonia would impact on cellular activities. There were no striking concerns in regards to a negative impact of the higher ammonia concentrations accumulated throughout this culture on cellular activities and protein quality. It is however important to note that in such cultures exponential growth ceased and cells entered stationary phase when the ammonia concentration reached 7 mM. At this time point not all of the glutamine had been consumed. This study has therefore demonstrated the importance of maintaining the accumulated ammonia concentration below 7 mM. The majority of publications reporting on the impact of ammonia on mammalian cell activities, particularly on product quality, have investigated this effect by means of incorporating high initial ammonia concentrations in the culture. This study has identified that the incorporation of high initial ammonia concentrations (5-15 mM) in a culture over-exaggerates the negative impact of high ammonia accumulation throughout the culture period on protein quality.

Chapter 4. Scale of CHO DP-12 cultures from shake flask to bench-scale minifors reactor

Abstract:

Conventional stirred-tank reactors are suitable for industrial production as they can be scaled up conveniently, provide a homogeneous environment for cell growth and proliferation and enable product quality to be controlled relatively easily. An investigation of the scale-up of CHO DP-12 cultures from 1 L Erlenmeyer shake flask platforms to 1.7 L minifors reactors for enhanced cell yields and recombinant protein production was conducted. Findings suggested that up-scaling does not improve cell or product yields with reduced growth rates and specific productivity rates of 11 % and 28 % , respectively observed in comparison to the 1 L Erlenmeyer shake flask studies. The effect of resulting shear stresses (4.5 dyn/cm^2) altered the metabolic profile of the cells cultured in the 1.7 L minifors reactor in comparison to 1 L Erlenmeyer shake flask cultures. Unfavourable glycolytic metabolism of glucose was noted resulting in a lactate molar yield in the ratio of 1 mole of glucose: 2 moles of lactate. It was concluded that the reduced galactosylation levels detected on the rIgG1 samples from the 1.7 L minifors reactor cultures were a direct effect of reduced nucleotide sugar donor synthesis in the form of UDP-GalNAc. The nucleotide sugar precursor for UDP-GalNAc, UTP, requires aspartate for synthesis. Reduced entry of pyruvate into the TCA cycle due to increased lactate dehydrogenase activity may have resulted in a depletion of available aspartate for UTP synthesis. This work has signified both the importance of cell adaptation to larger scale environments and also the requirement for cell protection from hydrodynamic shear stress

Abbreviations

APA	alginate-poly-L-lysine alginate
CHO	Chinese hamster ovary
CFD	Computational fluid dynamics
CO ₂	carbon dioxide
DO	dissolved oxygen
EDR	energy dissipation rate
GA	Golgi apparatus
GlcNAc	N-acetylgalactosamine

IgG1	Immunoglobulin G1
NaOH	sodium hydroxide
O ₂	oxygen
SEC	size exclusion chromatography
TCA	tricarboxylic acid
UDP-GalNAc	uridine di-phosphate N-acetylgalactosamine
UDP-GlcNAc	uridine di-phosphate N-acetylglucosamine
UDP-GNAc	uridine di-phosphate N-acetylhexosamine
UTP	uridine tri-phosphate

4.1 Introduction

The demand for increased yields in recombinant therapeutic proteins from mammalian cell culture systems is constantly rising, requiring cheaper, more efficient, cost effective process optimisations than those which currently exist. Antibodies are among the most expensive therapeutics being used, due to their associated low potencies thus requiring higher doses for therapeutic efficiency. Consequently large scale processes are required to produce 10-100 kg/year to provide for such demands. The biotechnological industry has responded to this need with advances being made in cultivation systems and their capacities, allowing for the upscaling of mammalian cell cultures resulting in increased volumetric productivity of 100-fold over the past decade as reviewed by Jain and Kumar, (2008).

In vitro cell culture techniques require a suitable cell cultivation system design allowing for adequate mass transfer of both nutrients and by-products and gases (oxygen (O₂) and carbon dioxide (CO₂)) and low shear stresses to the cells (Glacken et al., 1983). The use of small scale cell culture vessels such as Erlenmeyer shake flasks and stacked plates is associated with low-volume speciality applications such as vaccine production and gene therapies (Chu and Robinson, 2001). Working volumes are typically in the range of 10-500 ml (Zhang et al., 2010). Agitation rates applied to the shake flasks allow for the mixing of oxygen and nutrients within the culture medium and reduce the boundary layer at the construct surface (Jain and Kumar, 2008). Mammalian cells have relatively low demands for O₂ and consequently require lower specific power input, this being typically between 1-10 Wm⁻³. (Zhang et al., 2010). Typical cell densities achieved in shake flasks are in the range of 2.5-4 * 10⁶ cells/ml (Muller et al., 2004). The industrial scale production of therapeutic recombinant proteins utilising mammalian cell culture primarily involves the use of continuous stirred tank

bioreactors, which are associated with ease of scale-up and convenient means of control (Chu and Robinson, 2001). The popular stirred-tank bioreactor system consists of a vessel, pipes, valves, pumps, and a motor. Small stirred-tank bioreactor vessels are usually made of heat resistant silicate borate glass and tend to be used in research laboratories. Larger stainless steel vessels tend to be used for industrial applications, as reviewed by (Jain and Kumar, 2008). Stir-tank reactors are associated with many advantages making them suitable for industrial production. They can handle both anchorage-dependent and non-anchorage-dependent cell lines, can be scaled up conveniently, provide a homogeneous environment for cell growth and proliferation and enable product quality to be controlled relatively easily (Wang et al., 2005).

A range of operational parameters are required for consideration when scaling from low volume cell culture vessels, such as shake flasks, to larger scale platforms, such as stirred-tank reactors. Such parameters include temperature, pH, CO₂ pressure, dissolved oxygen (DO), and osmolality. The effects of alterations in such conditions on cellular activities, including quality of the produced recombinant protein, have all been extensively reviewed in Chapter 1. As the below experimental design focuses on the scale up of the cultures from 1 L Erlenmeyer shake flask to 1.7 L stirred-tank minifors reactor, the effects of shear stress caused to cells as a result of agitation and aeration systems employed in typical stirred-tank reactors will be a point of consideration for any alterations in the activities of the cells noted between cultivation in the two different vessels. Shear stress associated with agitation is as a result of turbulence within the liquid, whereas shear stress caused by aeration is due to gas-liquid interfacial effects, as reviewed by Zhang et al., (2010).

Shear stressed caused by extremely intense flows of liquid in the culture environment can be lethal resulting in the onset of cell death. At the physiological level, shear stresses are associated with the leakage of membrane ions, thus affecting the cytosolic pH of the cells (Ziegelstein et al., 1992). It has been estimated that a mean energy dissipation rate (EDR) of 10⁵-10⁶ W/kg is sufficient to disrupt animal cells (Zhang et al., 1993). The resistance level of Chinese Hamster Ovary (CHO) cells, in particular, to EDRs has been estimated at 10⁴-10⁵ W/kg (Ma et al., 2002). When in culture, it is estimated that cells are typically exposed to an average EDR of 0.01-0.15 W/kg (Nienow, 2006). It may therefore be concluded that the onset of cell death due to shear stress associated with flows of liquid is not an issue for consideration in mammalian cell culture systems. However there is cause for concern due to the vast amount of reports focusing on the non-lethal effects of culture associated shear

stresses on the physiological functions of cells. Such shear stresses have been associated with altered cell function, growth rate and viability, enhanced metabolism, inducement of transcriptional activators and alterations in the surface content of cellular receptors, as summarised by (Zhang, 2004). It is important to point out that alterations in recombinant protein production in CHO cells at 1 W/kg have been demonstrated. In such situations there was no alteration in cell morphology observed (Keane, 2003).

The avoidance of shear stress damage to cells through the application of minimal intensity of agitation in stirred-tank bioreactors for mammalian cell culture may in itself pose a risk in the optimisation of cellular activities. Low rates of agitation have been associated with inadequate levels of mixing and homogenisation of the culture environment, as reviewed by Zhang, (2004). The typical EDR value in a large scale 800 L aerated vessel is 0.035 W/kg 2.8-fold lower than that recommended for adequate mixing (Nienow et al., 1996). The low agitation rates in combination with low gas flowrates can affect DO gradients, pH control, dispersion of nutrients and waste products etc, as reviewed by Zhang et al., (2010). DO gradients may be observed in the bulk of the fluid and stagnant layer surrounding the bubbles. Inadequate gas dispersion has been associated with a build up of pCO₂ in the culture media causing the accumulation of levels which are inhibitory to cells. The use of alkaline solutions to control pH is brought about through addition of 2 M NaOH from the top of the stirred tank bioreactor. Sparged conditions result in pH excursions from the mean.

Shear stress damage to cells associated with gas-liquid interfaces may be caused at the regions of bubble generation, bubble coalescence and break-up in the bulk fluid, bubble rising area and bubble disengagement area, as reviewed by Zhang et al., (2010). Bubble associated cell damage is still not clear. Efforts have been made to quantify mammalian cell damage due to rising bubbles using Computational fluid dynamic (CFD) simulations. It was determined that at Reynolds numbers at which mammalian cells cultures typically operate, the high shear zone was confined to the wake of each bubble, with the EDR estimated to be around 4-8 W/kg for a bubble diameter greater than 1 mm.. Such EDR is expected to cause no cell damage (Koynov et al., 2007).

An experiment was designed which enabled an investigation of the scale-up from a 1 L Erlenmeyer shake flask platform to a 1.7 L minifors reactor. In keeping with the overall aim of the thesis, it was hypothesised that scale-up may be utilised as a means of achieving higher cell densities and enhanced recombinant protein production than that which was observed in the previous chapter for the recombinant CHO cell line DP-12, producing Immunoglobulin

G1 (IgG1). In the previous Chapter optimised growth, productivity and product quality were observed when cells were cultured in a concentration of 4 mM L-glutamine. This specific media composition was therefore applied to the scale up experiments in seeking to achieve further growth and productivity enhancements. Any alterations in cell growth, metabolism, productivity and product quality as a result of scale-up considerations would be identified.

4.2 Results

To evaluate the effect of scale up on the growth, metabolism and rIgG1 production and quality on the CHO DP-12 cell line, cells were cultured in both 1.7 L minifors reactors and 1 L Erlenmeyer shake flasks. The seeding density of the cells was approximately 0.3×10^6 cells/ml in a total culture volume of 300 ml in the Erlenmeyer shake flasks and 1.2 L in the minifors reactor. Cells were cultured in Excel CHO 325 PF media supplemented with 4 mM L-GLN, as determined to be the optimum L-GLN concentration for growth, production and rIgG1 quality in Chapter 3. Cultures conditions were maintained at 37 °C and 100 rpm agitation in both culture vessels. The minifors reactor was operated under conditions of 95 % DO by means of sparging with compressed air (flowrate of 0.06 L/min). The pH was also controlled at 7.2 through automatic addition of 2 M NaOH, via a feed line, or CO₂ sparging (through the headspace at a flowrate of 0.047 vvm). All cultures were completed in triplicate.

4.2.1 Effect on CHO cell growth and viability

To determine the effect of scale-up on the growth and viability of the CHO DP-12 cell line, samples were taken on a daily basis from cultures seeded at 0.3×10^6 cells/ml in both 1 L Erlenmeyer shake flasks and 1.7 L minifors reactors. Cell numbers were quantified using a haemocytometer and trypan blue exclusion method. The results of such cell counts are presented in terms of viable cell densities achieved, Figure 4.2.1.1, and viability over the culture period, Figure 4.2.1.2, are illustrated below. The maximum specific growth rate displayed by the cells during the exponential growth period and maximum viable cell density achieved are presented in Table 4.2.1.1

A similar growth pattern was displayed by the cells when cultured in 1 L Erlenmeyer shakes and in 1.7 L minifors reactors. Both modes of cultivation resulted in a lag period of 1 day before the entry of cells into exponential growth. Exponential growth was noted to last from days 1-4 for both cultures. A difference was noted however in the decline in viable cell density. Even though exponential growth at the maximum specific growth rate had ceased on

day 4 in 1 L Erlenmeyer shake flask cultures, cells did not enter a stationary phase until day 5. A significant fall off in viable cell numbers present did not occur until day 7. Logarithm plots of viable cell density over the culture period would suggest that cells were in stationary phase until day 7 (results not shown), the time at which the first decrease in viability 96 % to 81 % may be noted. Alternatively, viable cell number decreased rapidly 1 day after exponential growth had ceased for 1.7 L minifors reactor cultures. There were no further increases in viable cell number after cells had ceased growing at the maximum specific growth rate. Cells cultured in 1 L Erlenmeyer shake flasks displayed a maximum specific growth rate approximately 12 % higher than that displayed by cells cultured in 1.7 L minifors reactors. The combined result of an increased maximum specific growth rate and increase in viable cell number after exponential growth resulted in an increased biomass yield of 38 % when cells were cultured in 1 L Erlenmeyer shake flasks in comparison to 1.7 L minifors reactors.

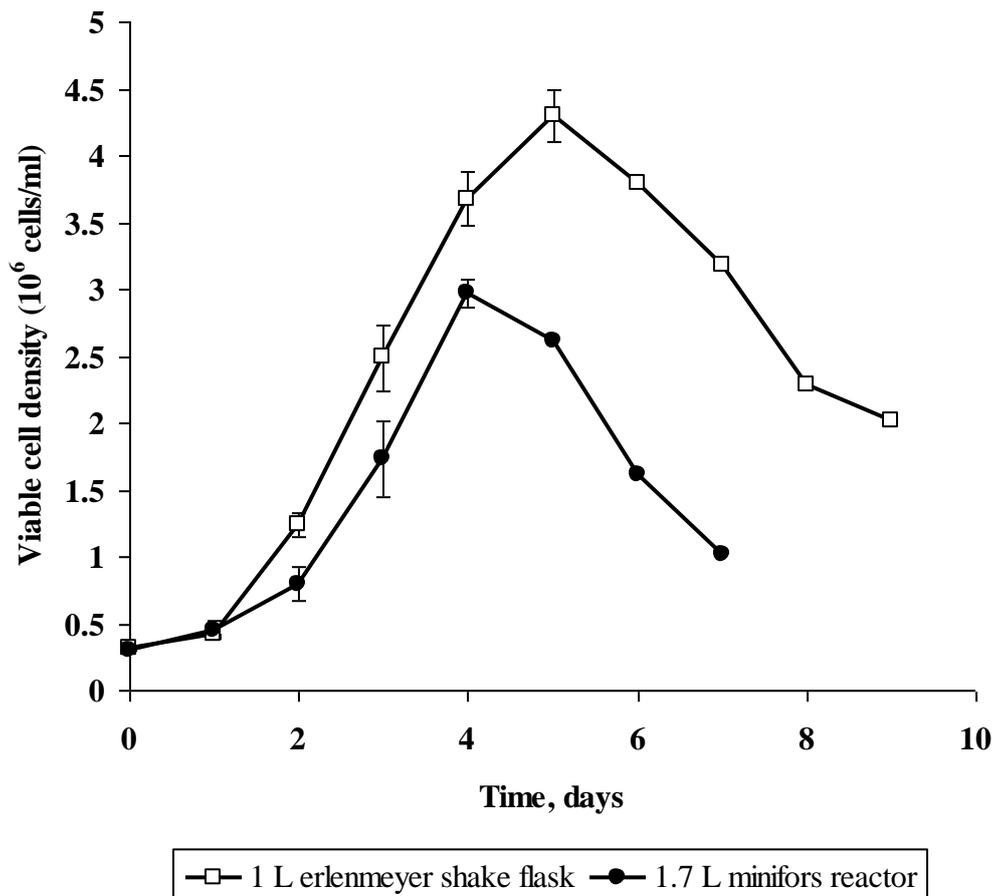


Figure 4.2.1.1: Growth of CHO-DP12 cells cultured in 1L Erlenmeyer shake flasks and 1.7 L minifors reactor. Cells were cultured as 37 C, 100 rpm agitation. Shake flask cultures were performed in un-vented shake flasks. Minifors reactor cultures were sparged with compressed air at a flowrate of 0.06 L/min. Agitation was achieved using a 3-blade pitched stainless steel impeller (45 ° angle). The pH was also controlled at 7.2 through automatic addition of 2 M NaOH, via a feed line, or CO₂ sparging (through the headspace at a flowrate of 0.047 vvm). Cell numbers were enumerated on a daily basis using trypan blue exclusion method and a Neubauer haemocytometer. Duplicate cell counts were performed. Both cultures conditions had a seeding viable cell density of 0.3×10^6 cells/ml. Values presented above are the average of triplicate cultures carried out in parallel +/- STD for both culture conditions tested.

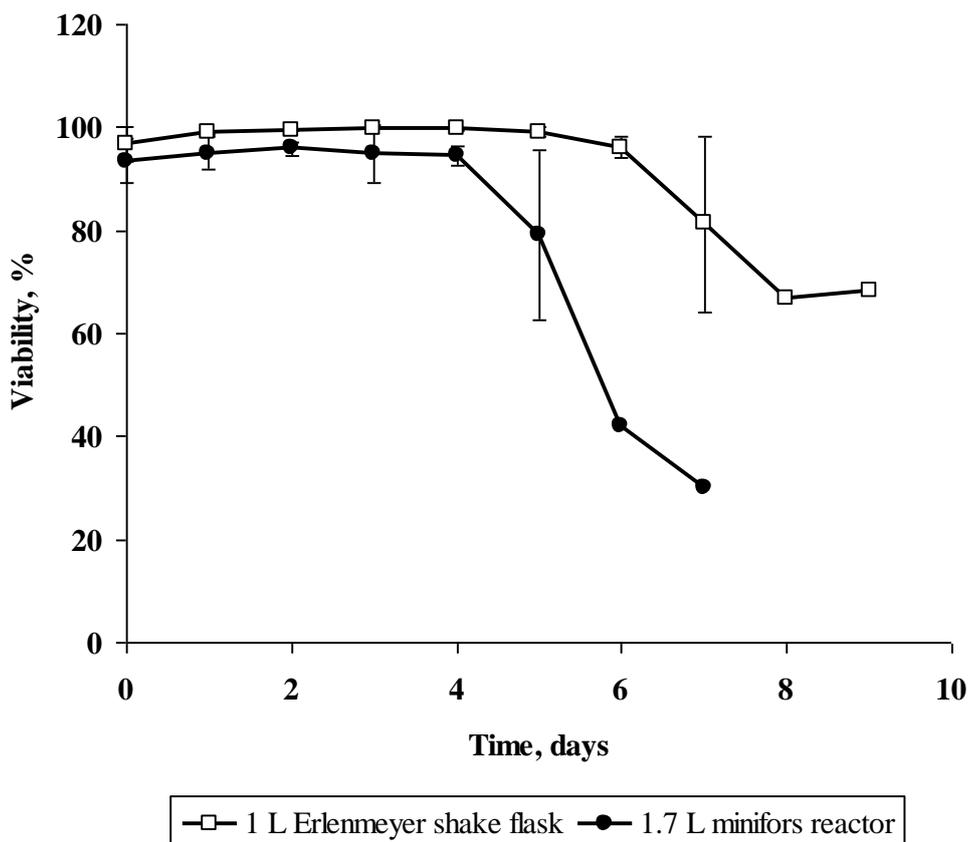


Figure 4.2.1.2: % viability of CHO cells cultures in 1L Erlenmeyer shake flasks and 1.7 L minifors reactor. % viability was determined by the enumeration of total and live cell numbers present in the culture using trypan blue exclusion method and Neubauer haemocytometer. % viability is expressed as a percentage of live cells over dead cells present. Cell counts were performed in duplicate and average % viability determined on a daily basis. Values presented above are the average of triplicate cultures carried out in parallel +/- STD, for both culture conditions tested.

Table 4.2.1.1: Calculated maximum specific growth rates displayed by cells cultured in 1 L Erlenmeyer shake flasks and 1.7 L minifors reactor over the exponential growth period. The growth rate was calculated from a plot of the logarithm of the viable cell density over the cultivation time (days). Values presented above are the average of triplicate cultures carried out in parallel +/- STD, for both culture conditions tested.

Culture	μ_{max} (day ⁻¹)	Max VCD (*10 ⁶ cells/ml)
1 L Erlenmeyer shake flask	0.725 +/- 0.013	4.295 +/- 0.191
1.7 L minifors reaction	0.645 +/- 0.037	3.12 +/- 0.27

4.2.2: Effect on cell metabolism

The concentration of the major carbon sources, glutamine and glucose, and their corresponding by-products, ammonia and lactate, were measured in cultures 1 L Erlenmeyer shake flask cultures and 1.7 L minifors reactor cultures (Figures 4.2.2.1 and 4.2.2.2). Samples were removed from cultures on a daily basis and centrifuged at 200 g for 5 minutes, followed by filtration of the supernatant (0.22 µm PTFE) in order to remove cells and culture debris. Glucose and lactate concentrations were determined using HPLC analysis, and glutamine and ammonia analysis was carried out using commercially available enzymatic assays kits, as described in Materials and Methods section 2.3.3. Determination of the concentration of the various metabolites enabled the calculation of the specific substrate uptake rates for each of the experimental cultures (Table 4.2.2.1).

An evaluation of the consumption profiles of glutamine for both 1 L Erlenmeyer shake flask cultures and 1.7 L minifors reactor cultures would suggest that the different modes of cultivation impacted on the utilisation of this carbon energy source by the cells. When cells were cultured in 1 L Erlenmeyer shake flasks, glutamine was exhausted by the end of the exponential growth phase, suggesting a cessation of maximum specific growth due to the depletion of this energy source. Alternatively glutamine was not depleted in 1.7 L minifors reactor cultures until cells went into decline phase, suggesting that this carbon source was not growth limiting. Although alterations were displayed on the requirement of glutamine for the maintenance of exponential growth of the cells, they did not however display significant differences in the rate of glutamine consumption. Ammonia accumulation was as expected for both cultures. As there were no significant differences in the specific rate of glutamine consumption displayed by the cells, it is also acceptable that the specific rate of ammonia production was also similar for the cells. A yield ammonia from glutamine of 1.07 mmol/mmol was determined for the shake flask cultures. The yield of ammonia from glutamine was not significantly different for the minifors reactor cultures, determined as 1.23 +/- 0.15 mmol/mmol.

All cultures were performed in ExCell CHO 325 PF media. The concentration of glucose in the commercially available media is expected to be 17.4 mM. The two modes of cultivation investigated also resulted in significantly different utilisation profiles of glucose by the cells. In 1 L Erlenmeyer shake flask cultures, glucose was exhausted on day 7, the point at which the first significant decline in cell number and % viability was noted. In 1.7 L minifors reactor cultures, glucose was completely consumed on day 4 of the culture period, signalling

the end of exponential growth. It would therefore appear that scale up of cultures from 1 L Erlenmeyer shake flask cultures to 1.7 L minifors reactor cultures did effect the growth limiting substrate required by the cells for exponential growth. A 2-fold increase in the specific glucose consumption rate was observed in 1.7 minifors reactor cultures in comparison to 1 L Erlenmeyer shake flask cultures during exponential growth. There was over a 2.2-fold increase in the concentration of lactate accumulated at the end of exponential growth by cells present in the 1.7 L minifors reactor in comparison to those cultured in the 1 L Erlenmeyer shake flasks. Similarly a 2.6-fold increase in specific lactate production rate was noted for the same culture conditions. A yield of lactate from glucose of 1.81 mmol/mmol was determined for the minifors reactor cultures. A significantly lower yield of 0.95 mmol/mmol was determined for the shake flask cultures. The results signify that although glucose did appear to be the growth limiting substrate in 1.7 L minifors reactor cultures, the inefficient glycolytic utilisation of glucose was evident under such conditions, resulting in high lactate concentrations. The cessation of maximum specific growth displayed by cells cultured in the 1.7 L minifors reactor may have been the result of a combination of glucose depletion and a build up of high lactate concentrations in the culture.

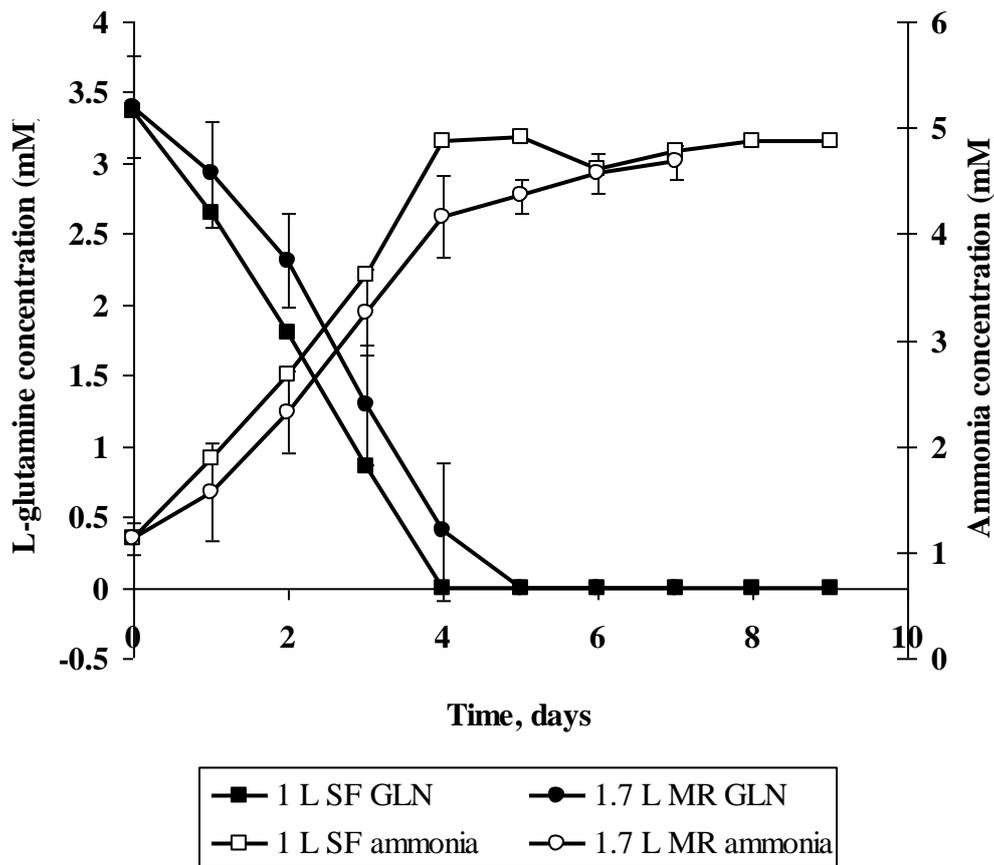


Figure 4.2.2.1: Concentration of glutamine and ammonia present in both 1 L Erlenmeyer shake flask cultures (SF) and 1.7 L minifors reactor cultures (MR) over the duration of the respective cultivation periods. The concentrations of glutamine and ammonia were determined offline using a commercial available enzymatic assay kit. Values presented above are the average of triplicate cultures carried out in parallel +/- STD, for each of the two conditions tested.

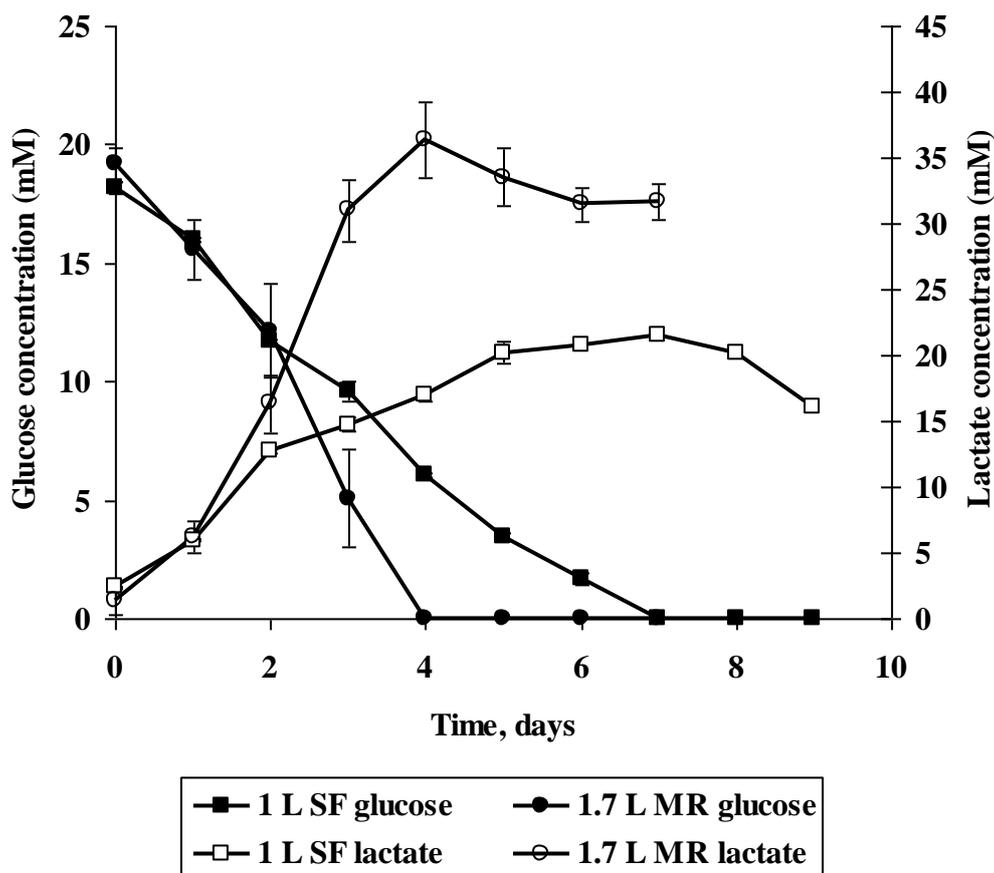


Figure 4.2.2.2: Concentration of glucose and lactate present in both 1 L Erlenmeyer shake flask cultures (SF) and 1.7 L minifors reactor cultures (MR) over the duration of the respective cultivation periods. The concentrations of glucose and lactate were determined offline by HPLC analysis using Supelco H column. Values presented above are the average of triplicate cultures carried out in parallel +/- STD, for each of the two conditions tested.

Table: 4.2.2.1: Calculated rates of consumption and production of key metabolites as determined by offline analysis throughout the culture period. Cell specific consumption and production rates of the key metabolites were determined during the exponential growth phase. Yields of by-product on metabolite were calculated over the cultivation period. The results presented below are the average of that calculated for triplicate cultures carried out in parallel for each of the culture conditions tested.

	1 L Erlenmeyer shake flask	1.7 L minifors reactor
Glucose consumption rate (mM/day)	3.20 +/- 0.033	5.37 +/- 0.39
Glutamine consumption rate (mM/day)	0.89	0.81 +/- 0.057
Lactate production rate (mM/day)	3.49 +/- 0.17	10.54 +/- 0.40
Ammonia production rate (mM/day)	0.902	0.78 +/- 0.04
q_{glucose} (mmoles/ 10^6 cell/day)	$8.35 * 10^{-4} +/- 5 * 10^{-5}$	$1.69 * 10^{-3} +/- 1.00 * 10^{-4}$
$q_{\text{glutamine}}$ (mmoles/ 10^6 cell/day)	$2.26 * 10^{-4}$	$3.00 * 10^{-4} +/- 3.31 * 10^{-5}$
q_{lactate} (mmoles/ 10^6 cell/day)	$1.15 * 10^{-3} +/- 8.59 * 10^{-5}$	$3.07 * 10^{-3} +/- 3.3 * 10^{-4}$
q_{ammonia} (mmoles/ 10^6 cells/day)	$3.11 * 10^{-4}$	$3.46 * 10^{-4} +/- 2.74 * 10^{-5}$
$Y_{\text{lactate/glucose}}$ (mmol/mmol)	0.95 +/- 0.11	1.81 +/- 0.09
$Y_{\text{ammonia/glutamine}}$ (mmol/mmol)	1.07	1.23 +/- 0.15

4.2.3: Effect on rIgG1 production

To evaluate the effect of the two modes of cultivation on rIgG1 production, media samples were removed from the suspension cultures on a daily basis from day 4 onwards and on day 5 onwards from the encapsulated cultures. As explained in Section 2.3.6 of the Materials and Methods section, rIgG1 was purified from media samples removed from the

cultures after centrifugation at 200 g for 5 minutes to remove cells. rIgG1 present in the media was purified using Protein A Nab spin columns and quantified using the nanodrop at A 280 nm.

Evaluation of the concentration of rIgG1 produced by cells cultured in the two different modes, as presented in Figures 4.2.3.1 and 4.2.3.2 below, suggested that enhanced recombinant protein production was observed when cells were cultured in 1 L Erlenmeyer shake flasks in comparison to 1.7 L minifors reactors. Investigation of the specific production rate displayed by the cells cultured in the 1 L Erlenmeyer shake flask, presented in table 4.2.3.1, suggests that this is not due to the increased number of cells present producing the protein. Both enhanced volumetric productivity and enhanced specific productivity (1.4-fold) of the rIgG1 was observed for the cultivation of cells in the 1 L Erlenmeyer shake flasks. Such observations cumulated in a 2.2-fold higher concentration of rIG1 present at the end of the stationary phase for the 1 L Erlenmeyer shake flask cultures in comparison to that observed in 1.7 L minifors reactor cultures.

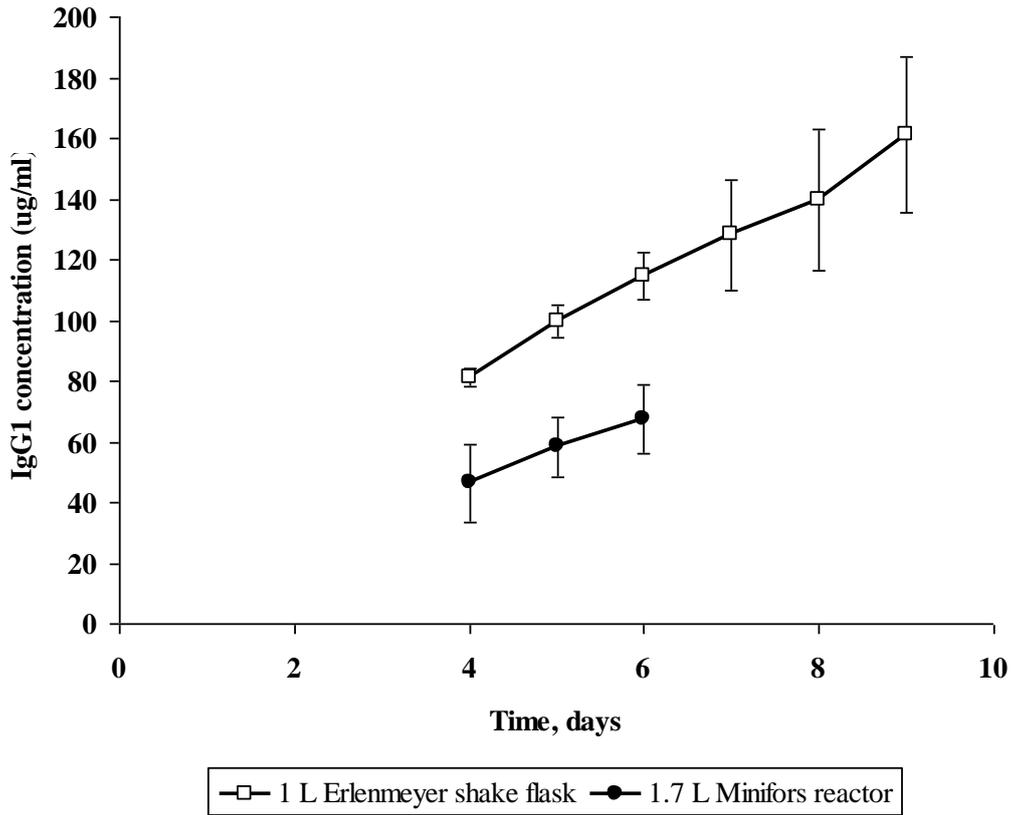


Figure 4.2.3.1: Concentration of rIgG1 purified from cell free media samples harvested from both 1 L Erlenmeyer shake flask cultures and 1.7 L minifors reactor cultures from day 4 onwards. rIgG1 present in the media was purified using commercially available protein A/G Nab spin columns and quantified using the nanodrop A280 nm in duplicate which the average value recorded. Values presented above are the average of triplicate cultures carried out in parallel at both scales +/- STD.

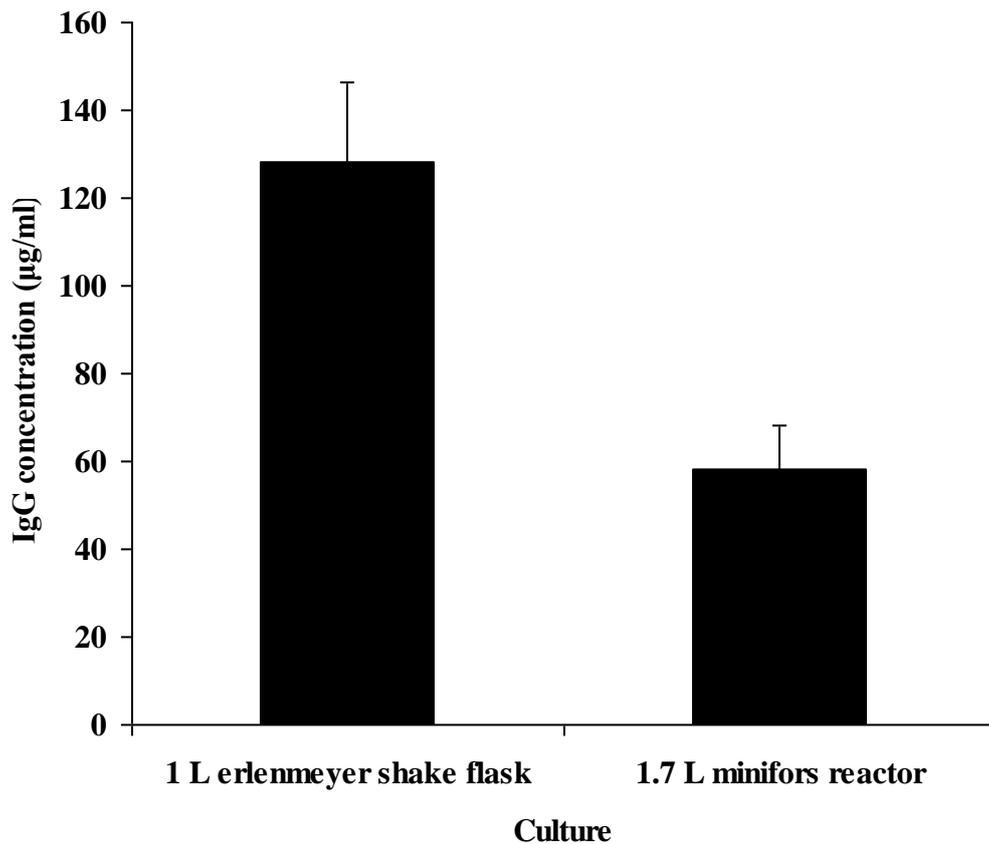


Figure 4.2.3.2: Concentration of rIgG1 purified from cell free media samples harvested from 1 L Erlenmeyer shake flask cultures and 1.7 L minifors reactor cultures at the end of the stationary growth period. rIgG1 was purified using Protein A/G spin columns and quantified using nanodrop A280 nm. Samples were quantified in duplicate with the average value recorded. Values presented above are the average of triplicate cultures carried out in parallel +/- STD.

Table 4.2.3.1: Calculated rIgG1 production rates and cell specific production rates for cells cultured in 1L Erlenmeyer shake flask and 1.7 L minifors reactors. The specific production rate was calculated over the exponential growth phase for all cultures. The results presented below are the average of triplicate cultures, +/- STD, carried out in parallel for the two culture conditions tested.

Culture	IgG1 production rate µg/ml/day	q_{IgG1} µg/10 ⁶ cells/day
1 L erlenmeyer shake flask	16.787	5.16 +/- 0.29
1.7 L minifors reactor	10.522	3.74 +/- 0.23

4.2.4: Effect on rIgG1 quality

To assess the affect of scale-up from 1 L Erlenmeyer shake flasks to 1.7 L minifors reactors on the quality of rIgG1 produced, both glycan and aggregation analysis were completed on the purified rIgG1 samples harvested from both the shake flask and minifors reactor cultures at both the end of the exponential and stationary growth phases Samples harvested from the shake flask and reactor cultures were subjected to N-glycan release and labelled as outline in Materials and Methods section 2.3.7.1. HILIC characterisation of the released and labelled N-glycans produced a chromatogram consisting of 8 distinctly separated peaks. Each of the peaks was assigned a distinctive glucose units (GU) value by comparison to the retentions times of the standard A-AB labelled external dextran. Examination of exoglycosidase treated samples allowed for the corresponding number and linkage of the component monosaccharide units represented by each GU value to be elucidated, as described in Materials and Methods section 2.3.7.3 Aggregation analysis of the purified samples was carried out using size exclusion chromatography (SEC)

Figure 4.2.4.1 below illustrates the relative % of the different glycans present on rIgG1 samples harvested from the cultures at the end of the stationary growth phase. It is important to note that the relative % of glycans present on IgG1 samples harvested at the end of the exponential and stationary growth phases did not vary significantly for each of the culture conditions investigated. Irrelevant of the mode of cultivation, all cultures displayed a similar trend in the relative % of glycans present. The earliest form of N-glycosylation of rIgG1 is denoted by M5, F6A1/A2 and F6A2. The highest relative percentage of glycans present on the rIgG1 samples were the early glycoforms, predominantly F6A2. The galactosylated glycoforms are denoted by F(6)A2[6]G(4)1 and F(6)A2[3]G(4)1, which contain 1 galactose unit on either of the outer GlcNAc arms, and F(6)A2G2, which contains a galactose residue on each of the outer GlcNAc arms. A2G2 contains galactose residues on each outer arm but if not fucosylated. Of the galactosylated glycoforms present, the F(6)A2[6]G(4)1 were the predominant form in all cultures, independent of the mode of cultivation employed.

Similar to the analysis completed in Chapter 3; Section 3.2.4, a more in depth evaluation of the relative % of each type of glycoform present was completed and did indicate significant differences in the levels of glycosylation between cultures. Figure 4.2.4.2, below, illustrates the relative % of F(6)A1/A2 glycan structures released from rIgG1 samples harvested from shake flask and reactor cultures. An average of 10 % increase in the relative % of this glycan was present on rIgG1 samples harvested from minifors reactor cultures in comparison to

shake flask cultures. This fucosylated N-glycan, containing either one or two GlcNAc residues is one of the earliest form of glycosylation arising from initial processing in the Golgi apparatus (GA). The proportion of this glycoform may suggest a lack of further processing of glycans on proteins produced by cells cultured in 1.7 L minifors reactor in comparison to that which is observed when cells are cultured in 1 L Erlenmeyer shake flasks. This statement is confirmed by the presence of a lower relative % of glycan structures containing one or two galactose residues present on proteins harvested from the reactor cultures in comparison to the shake flask cultures, as noted in Figures 4.2.4.3 and 4.2.4.4. A 18 % lower relative % of, F6A2[6]G(4)1, and a 31 % lower relative % of F6A2G2 was noted for glycans released from rIgG1 harvested from 1.7 minifors reactor samples in comparison to 1 L Erlenmeyer shake flask samples. The level of sialylation did not appear to be significantly affected by the scale up of the cultures. It may therefore be proposed that the scale-up of cultures from 1 L Erlenmeyer shake flask cultures to 1.7 L minifors reactor cultures effects the complex processing of glycan structures present on recombinant proteins at the galactosylation level. Aggregation analysis confirmed that over 99 % of the rIgG1 harvested from 1 L Erlenmeyer shake flask cultures and 1 L minifors reactor cultures was in monomeric form. These results suggest that neither the culture conditions investigated nor the resulting glycoform profile of the recombinant protein affected the aggregation state of the protein.

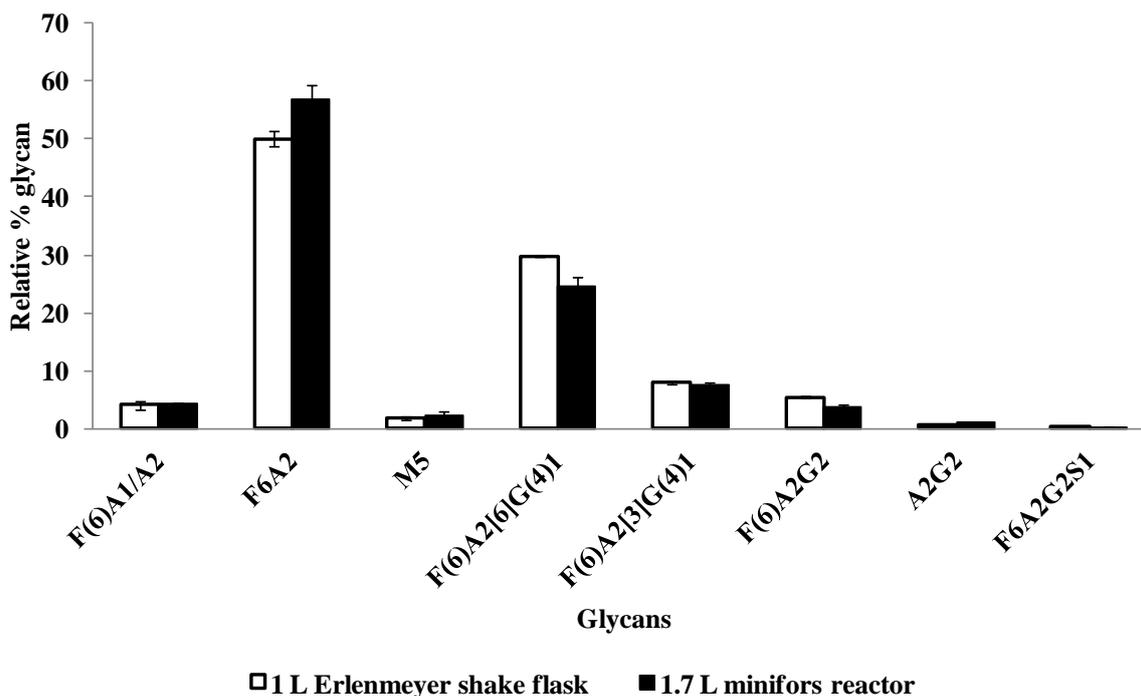


Figure 4.2.4.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from 1 L Erlenmeyer shake flask and 1 L minifors reactor studies at the end of the stationary growth phase. N-glycans were released from the rIgG1 samples by means of PNGaseF digestion. Samples for HILIC analysis were labelled by adding 5 μ l of 2AB labelling solution. The retention time of the glycan peaks was compared with an external A-AB labelled dextran standard for structural elucidation by converting the retention time of the peaks into GU. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for each condition tested.

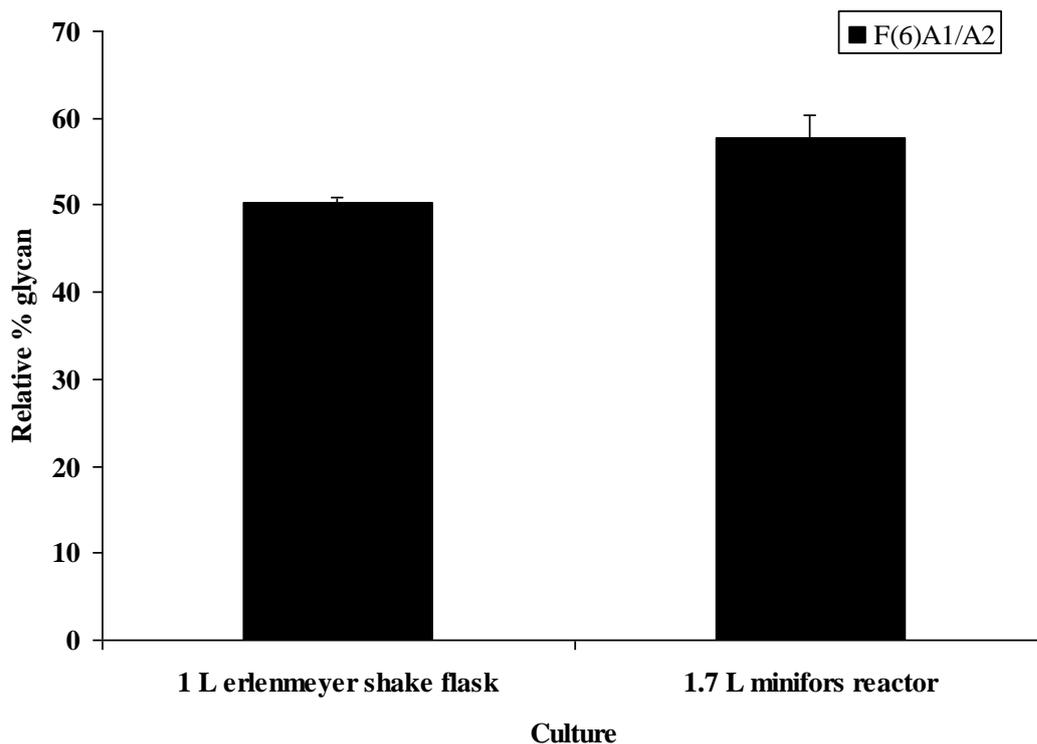


Figure 4.2.4.2: Relative % of F(6)A1/A2 glycan forms detected on rIgG1 samples harvested from 1 L Erlenmeyer shake flask and 1 L minifors reactor studies at the end of the stationary growth phase, as detected using HILIC following PNGaseF release and 2-AB labelling. Results are directly comparable results to those found for the analysis of samples from the end of the exponential growth period. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for the two culture conditions tested +/- STD

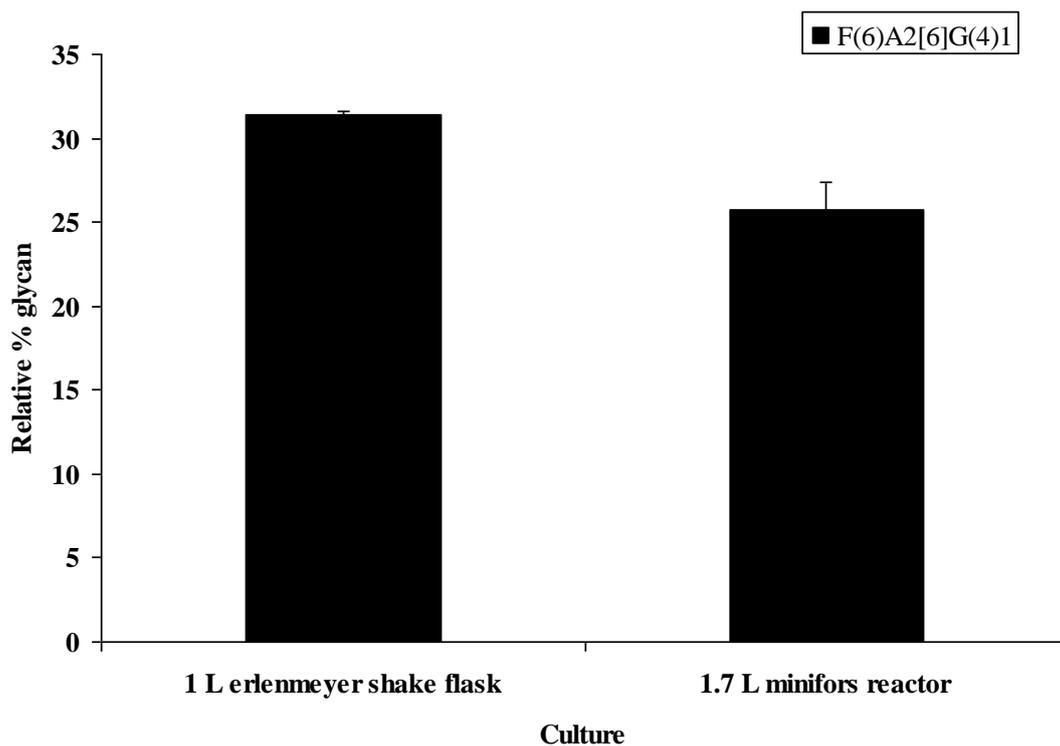


Figure 4.2.4.3: Relative % of F(6)A2[6]G(4)1 glycan forms detected on rIgG1 samples harvested from 1 L Erlenmeyer shake flask and 1 L minifors reactor studies at the end of the stationary growth phase, as detected using HILIC following PNGaseF release and 2-AB labelling. Results are directly comparable results to those found for the analysis of samples from the end of the exponential growth period. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for the two culture conditions tested +/- STD.

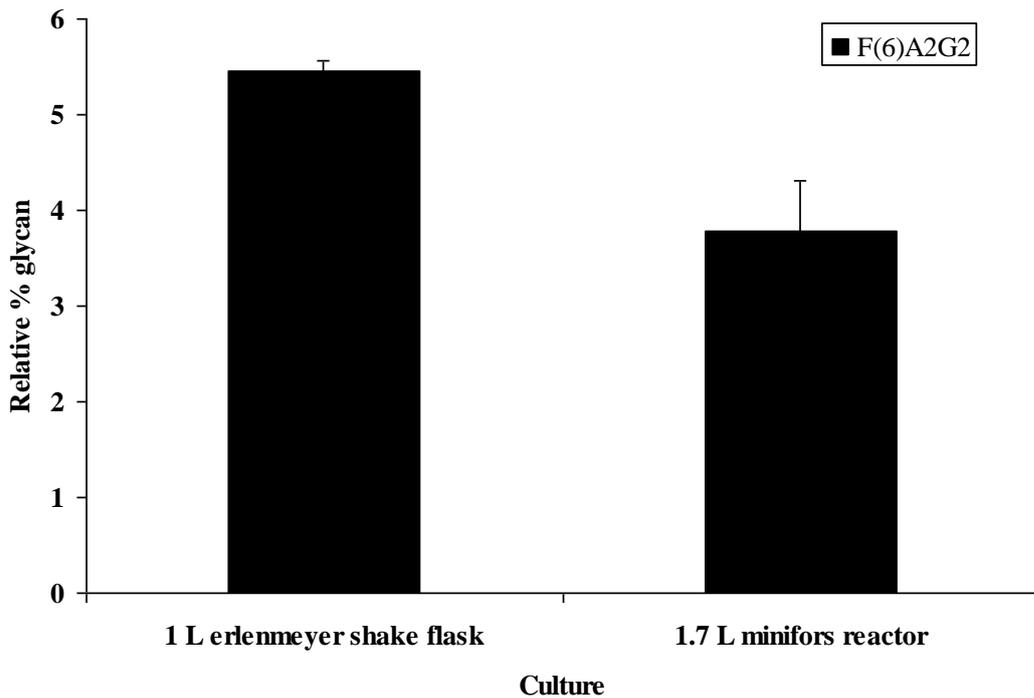


Figure 4.2.4.4: Relative % of F(6)A2G2 glycan forms detected on rIgG1 samples harvested from 1 L Erlenmeyer shake flask and 1 L minifors reactor studies at the end of the stationary growth phase, as detected using HILIC following PNGaseF release and 2-AB labelling. Results are directly comparable results to those found for the analysis of samples from the end of the exponential growth period. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for the two culture conditions tested +/- STD.

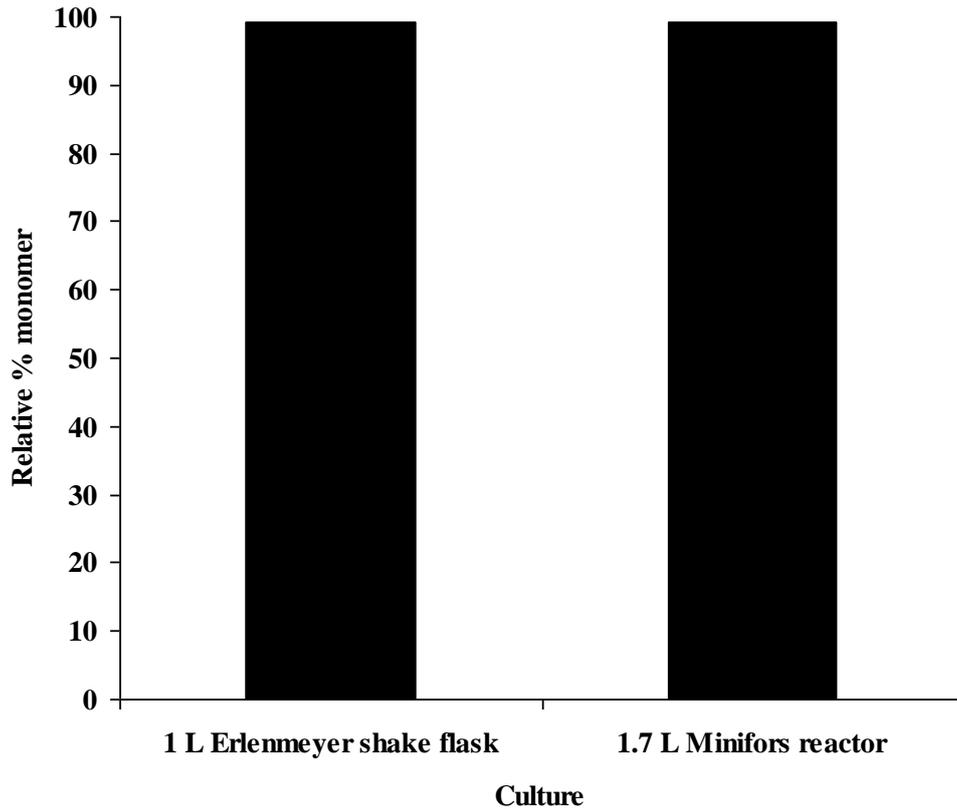


Figure 4.2.4.5: Relative % of monomeric form of rIgG1 samples as detected by SEC. IgG1 samples were harvested from cultures performed in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. The above results are based on the analysis of samples harvested at the end of the stationary growth period. The results for samples harvested over the culture period in each of the cultures are directly comparable (data not shown). IgG1 samples were analysed in duplicate. The results are presented as the average of triplicate cultures performed for the two culture conditions tested +/- STD.

4.2.5: Estimation of maximum shear stress under stirred conditions

It is possible to quantitatively determine the maximum shear stress experienced by the CHO DP-12 cells cultivated in the 1.7 L minifors reactor platform employing both mechanical aeration and agitation. Air was sparged into the reactor at a flowrate of 0.06 L/min. A 3 blade pitched stainless steel impeller (45 ° angle) was employed for agitation of the reactor.

The resulting EDR, denoted as ε_t , resulting from aeration and agitation was calculated by the sum of the calculated energy dissipation rate from sparging ε_s and the energy dissipation

rate from the impeller under sparged conditions ε_{is} . Such calculations were made utilising the parameters listed in Table 2.2.2.1 and Equations 2.2.2.1.2 – 2.2.2.1.12 in Materials and Methods sections 2.2.2 and 2.2.2.1, respectively.

Once the overall EDR from aeration and agitation was calculated, it was possible to estimate the overall maximum shear stress, τ_{max} , on the cells using Equation 2.2.2.1.1 from Materials and Methods section 2.2.2.1. Table 4.2.5 below gives the calculated ε_s , ε_{is} and overall EDR (ε_t), all in W/kg. The τ_{max} is also presented in the table below dyn/cm².

Table 4.2.5: Maximum shear stress exerted on the cells under conditions of mechanical agitation and aeration in 1.7 L minifors reactor. Overview of the energy dissipation rates from sparging ε_s , impeller agitation under conditions of sparging ε_{is} , overall EDR ($\varepsilon_t = \varepsilon_{is} + \varepsilon_s$) and maximum shear stress on cells τ_{max} .

ε_s (W/kg)	ε_{is} (W/kg)	ε_t (W/kg)	τ_{max} (dyn/cm ²)
0.0024	0.0058	0.0082	4.5

4.3 Discussion

An experiment was designed which enabled an investigation of the scale-up from a 1 L Erlenmeyer shake flask platform to a 1.7 L minifors reactor. In keeping with the overall aim of the thesis, it was hypothesised that scale-up may be utilised as a means of achieving higher cell densities and enhanced recombinant protein production than that which was observed in the previous chapter for the recombinant CHO cell line DP-12, producing IgG1. Typical cell densities achieved in shake flasks are in the range of $2.5-4 \times 10^6$ cells/ml (Muller et al., 2004). The industrial scale production of therapeutic recombinant proteins utilising mammalian cell culture primarily involves the use of continuous stirred tank bioreactors, which are associated with ease of scale-up and convenient means of control (Chu and Robinson, 2001) In the previous chapter optimised growth, productivity and product quality were observed when cells were cultured in a concentration of 4 mM L-glutamine. This specific media composition was therefore applied to the scale up experiments in seeking to achieve further growth and productivity enhancements.

The overall results of the effect of scale-up from a 1 L Erlenmeyer shake flask to 1.7 L minifors reactor was a reduction in cell growth, denoted by an 11 % reduction in the

maximum specific growth rate and a 27 % reduction in the viable cell density achieved. The reduction in cell yield was as a result of the combined effect of the reduced maximum specific growth rate and also the fact that cells cultured in the 1 L Erlenmeyer shake flask continued to grow for one extra day after maximum growth had ceased. Cells cultured in 1.7 L minifors reactor only maintained the maximum cell yield achieved for that condition for approximately one day after maximum growth had ceased. Lee et al., (2005) also reported a 27 % decrease in the maximum cell density achieved between shake flask and STR cultures under batch cultivation conditions. Reductions in the final yield of recombinant protein produced were also noted in this current study for cells cultured in the 1.7 L minifors reactor, in comparison to those cultured in the 1 L Erlenmeyer shake flask. The reduced yield was as a result of the lower cell numbers producing the protein, and also a noted 28 % reduction in the specific production rate displayed by the cells. In a similar study Lee et al., (2005) reported on a 35 % reduction in the maximum specific production rate when cells were cultured in 1 L (working volume) STR in comparison to shake flask cultures for a recombinant CHO cell line. The scale-up of the cultures did also impact on the quality of the recombinant protein produced, in terms of the level of galactosylation achieved. rIgG1 harvested from 1.7 L minifors reactors had an 31 % reduction in the relative percentage of fully galactosylated N-glycan structures, F6A2G2, and an 18 % reduction in the relative % of N-glycan structures containing 1 galactose residue, F6A2[6]G(4)1, in comparison to the rIgG1 harvested from 1 L shake flask cultures.

Culture conditions which affect cell growth and productivity, such a temperature and pH were maintained as consistent as possible between the two culture conditions. Significant differences in operational parameters between the two cultivation platforms exist in the agitation of the minifors reactor and it's gassing, as explained in Materials and Methods section 2.2.2. The minifors reactor was operated under conditions of 95 % dissolved oxygen (DO) by means of sparging with compressed air (flowrate of 0.06 L/min). The pH was also controlled at 7.2 through automatic addition of 2 M NaOH, via a feed line, or CO₂ sparging (through the headspace at a flowrate of 0.047 vvm). Three things to consider in scaling up of vessels which effect cellular activities: (1) shear stress associated with turbulence in the liquid brought about by agitation (2) the presence of DO gradients , inadequate pH control and nutrient dispersion brought about by inadequate mixing, again associated with agitation (3) shear stresses associated with the gas to liquid interface. Shear stress associated with agitations and aeration can have both lethal, resulting in cell death, and non-lethal, altering

growth, metabolism and productivity profiles of the cells, effects. It may be suggested that the reduced growth and productivity demonstrated by the cells in the minifors reactor was as a result of shearing stresses brought about by the conditions. It has been estimated that a mean EDR of 10^5 - 10^6 W/kg is sufficient to disrupt animal cells (Zhang et al., 1993). The resistance level of CHO cells, in particular, to EDRs has been estimated at 10^4 - 10^5 W/kg (Ma et al., 2002). Variations in the susceptibility of mammalian cells to mechanical agitation have been varied. Chisti, (1993) scaled up hybridoma cells from 20 L to 300 L and found that the cells were fairly resistant to high impeller speeds. Reynolds numbers, submerged aeration, grinding action of the mechanical seals and support bearings of the magnetic agitators did not cause much damage to the cells up to 300 L. In relation to the shear stress associated with gassing, a maximum EDR of 10^3 - 10^4 W/kg was calculated due to bubble rupture of small bubble diameter (less than 2 mm) (Boulton-Stone and Blake, 1993; Garcia-Briones and Chalmers, 1994). Kioukia et al., (1992) found that the most important region for cell damage was the bubble disengagement region and the more frequently cells passed through that region, the greater the cell damage was. pH perturbations brought about by a combination of sparging and NaOH addition at the top of the reactor result in hybridoma cell death when pH is below its mean value (Osman et al., 2002). Low shear stress levels, EDR values of 1 W/kg, have also been noted to affect the productivity of recombinant CHO cells. In protein-free media, without the presence of protectants, productivity ceased from CHO cells when a shear stress of 1 W/kg was generated in a flow chamber. In this current study the EDR from the combination of sparging and impeller agitation was calculated at approximately 0.0082 W/kg, which is considerably lower than the EDR which has been noted to impact on cell growth and productivity in the above mentioned studies. Increasing shear stresses, with EDR in the range of 0.05-8 W/kg reduced productivity from 100 % to 49 % respectively for the same cell line (Keane et al., 2003). The overall maximum calculated shear stress on the cells, τ_{\max} , was however calculated as 4.5 dyn/cm^2 . A study performed by Fernandes-Platzgummer et al., (2011) investigated the effect of increases in maximum shear stress values, from 1.2-3.0 dyn/cm^2 , on the growth of mammalian cells. The mouse embryonic stem (mES) cells in the study demonstrated inefficient cell expansion when cultured in the presence of a shear stress level of 3.0 dyn/cm^2 in comparison to cells cultivated in the presence of 1.2 and 2.0 dyn/cm^2 , with reduced viability also being noted on day 2 in the 3.0 dyn/cm^2 cultures. Shear stresses created by scale up have been noted to affect the metabolism of cells. Under most culture conditions, cells predominantly take up glucose as a carbon source, which is

catabolised via different pathways with a large proportion converted to lactate (Chen et al., 2012). The tolerance levels towards lactate seem to be cell line specific and vary widely (Hassell et al., 1991). The effect of shear stress is somewhat varied in the response of cells by means of altered glucose consumption and lactate production, Keane et al.,(2002) reported that a shear stress in the range of 1-8 W/kg resulted in a concurrent increase in glucose uptake rate from 115% to 142% in compared to control cultures with no shear. Lactate productivity was decreased from 92 to 50 % over the same range. These results do not comply with those presented by Shiragami and Unno, (1994), who found metabolite production increased with exposure to moderate shear stress with dissipation energy of the flowing medium. The effects of non-lethal levels of shear stress on the metabolism of various cell types were established by examining the induction of the transcriptional activator c-fos (Ranjan et al., 1996). CHO cell response to 25 W/kg shear stress was within minutes. The mechanism of action on intracellular responses has been associated with involvement of intracellular Ca^{++} in a membrane event linked to the activation of the phospholipase C pathway (Levesque et al., 1989, Wiesner et al., 1997). However it has also been associated with the activation of a specific GTP-binding (G protein), activated by shear stress within 1 sec of onset in human cells (Gudi et al., 1996). The impact of scale-up from 1 L Erlenmeyer shake flask to 1.7 L minifors reactor is portrayed by an altered glucose consumption and lactate production profile. The specific consumption and production profiles of glutamine and ammonia were similar for both culture conditions investigated. Glucose appeared to be the growth limiting substrate for cells cultured in the 1.7 L minifors reactor. A 2-fold increase in the glucose consumption rate was observed over the exponential growth phase in comparison to that observed when cells were cultured in the 1 L Erlenmeyer shake flasks. A 2.6-fold increase in the specific lactate production rate was also observed for the cells cultured in the minifors reactor. Cells cultured in 1.7 L minifors reactors underwent higher rates of glycolysis resulting in yields of lactate from glucose, determined as 1.81 mmol/mmol. Cells cultured in 1 L Erlenmeyer shake flasks displayed significantly reduced yields of lactate, remaining at 0.95 mmol/mmol. It may therefore also be proposed that the effects of scale-up in cell and protein yields may also be a direct result of the alterations in the metabolic profiles of the cells between the two culture platforms. High lactate concentrations, induced by the addition of 60 mM sodium lactate have been noted to inhibit growth in CHO cells by 25 % (Lao and Toth, 1997). At the end of the cultivation period in hybridoma cultures, the concentration of lactate was noted to be 63 mM which was thought to be associated with the onset of cell

death (Zhang et al., 2004). The maximum lactate accumulation at the end of the exponential phase in the minifors reactor cultures was 36 mM. Such a level is not suggested to be inhibitory of cell growth, but may in turn have caused cell stress which did not occur in the shake flask studies, resulting in reduced cell growth and productivity.

The altered metabolic glucose patterns may also serve as a possible reasoning for the reduced levels of galactosylation noted on the final product. As explained in Chapter 1, galactose residues are transferred from the pool of nucleotide sugar donors uridine di-phosphate N-acetylgalactosamine (UDP-GalNAc) present in the Golgi apparatus (GA), onto the N-acetylgalactosamine (GlcNAc) residue of an emerging glycan chain of a newly synthesised protein. This process is very much dependent upon both the action of galactosyl transferase enzymes and also the pool of free nucleotide sugar donors in the GA. A limitation in the concentration of glucose has been noted to reduce the pool of nucleotide sugar donors formed by means of reducing the intracellular concentration of nucleoside triphosphates, uridine triphosphate (UTP) (Nyberg et al., 1999). In the 1.7 L minifors reactor studies, the concentration of glucose was not limiting in comparison to that present in the 1 L Erlenmeyer shake flask studies. However the fact that the majority of glucose present was converted to lactate suggests that a reduction in the amount of pyruvate entering the tricarboxylic acid (TCA) cycle was likely to occur. A key precursor for UTP synthesis is aspartate. The importance of the TCA cycle in providing aspartate for UTP synthesis has been demonstrated by DeFrancesco et al., (1976), in which Chinese hamster lung fibroblasts could not readily take up exogenously supplied aspartate and demand for intracellular aspartate relied on its production from oxaloacetate, an intermediate of the TCA cycle. As pyruvate directly feeds into the TCA cycle, a reduction in pyruvate due to increase lactate production may result in a reduction in available aspartate for UTP synthesis. A reduction in UTP synthesis would subsequently reduce the intracellular pool of uridine di-phosphate N-acetylhexosamines (UDP-GNAc) such as UDP-GalNAc. There was no apparent reduction in the concentration of uridine di-phosphate N-acetylglucosamine (UDP-GlcNAc) residues due to similar relative % of GlcNAc containing glycans, such as F6A2, present on rIgG harvested from both culture conditions studied. It may therefore also be suggested that perhaps only the further processing steps, such as galactosylation, may have been affected by a reduced pool of UTP.

4.4 Conclusion:

An investigation of the scale-up of CHO DP-12 cultures from 1 L Erlenmeyer shake flask platforms to 1.7 L minifors reactors for enhanced cell yields and recombinant protein production was conducted. In this study scale-up was not shown to enhance cell or product yields with reduced growth rates and specific productivity rates of 11 % and 28 % , respectively observed in comparison to the 1 L Erlenmeyer shake flask studies. The maximum shear stress exerted on the cells in the 1.7 L minifors reactor was calculated as 4.5 dyn/cm², due to a combination of sparging and impeller agitation. The altered growth and productivity profiles of the cells in the minifors reactor in comparison to the shake flask platform were due to the non-lethal effects of such a shear stress on the cells. The effect of resulting shear stresses altered the metabolic profile of the cells cultured in the 1.7 L minifors reactor, with enhanced glucose consumption and lactate production observed in comparison to 1 L Erlenmeyer shake flask cultures. Unfavourable glycolytic metabolism of glucose was noted resulting in a lactate molar yield in the ratio of 1.81 mmol/mmol. It was concluded that the reduced galactosylation levels detected on the rIgG1 samples from the 1.7 L minifors reactor cultures were a direct effect of reduced nucleotide sugar donor synthesis in the form of UDP-GalNAc. The nucleotide sugar precursor for UDP-GalNAc, UTP, requires aspartate for synthesis. Reduced entry of pyruvate into the TCA cycle due to increased lactate dehydrogenase activity may have resulted in a depletion of available aspartate for UTP synthesis.

A step forward from this study could include an investigation of the optimisation of bioreactor parameters in the 1.7 L minifors reactor platform, in order to determine if the adverse growth, productivity and product quality noted to occur, in comparison to shake flask cultures, could be improved. Optimisation of bioreactor parameters are typically the measures taken at an industrial level. This current study has focused on the effect of increased shear stress brought about by mechanical aeration and agitation in 1.7 L minifors reactor platforms in comparison to shake flask cultures. Although the maximum shear stress calculated in the reactor, 4.5 dyn/cm², was at a level which was previously determined to disrupt the growth expansion of a stem cell line under stirred conditions (Fernandes-Platzgummer et al., 2011), the EDR were not at the levels which have previously been reported to disrupt CHO cell growth, in both a lethal or non-lethal manner, as noted in the discussion section. In order to optimise cellular activities in the reactor and overcome the adverse growth, productivity and metabolism noted it would be necessary to investigate all parameters which were different

between the 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platform, which include:

- 1) shear stress (agitation and aeration), which has already been investigated in this Chapter.
- 2) The oxygen transfer rate (OTR). The importance of the OTR in the supply of oxygen to cells in culture has been described in Chapter 1, Section 1.2.4. It is influenced by several parameters including the air flowrate, bubble size, agitation rate and liquid properties (Morão et al., 1999). Variations in the volumetric oxygen mass transfer coefficient, (k_{La}), have been noted for airflow rates ranging from 100-300 cm^3/min (Fernandes-Platzgummer et al., 2014). The air flowrate used in this current study was 60 cm^3/min .
- 3) The sparging of the reactor with CO_2 (shake flask cultures were previously conducted in a non-gased incubator in capped shake flasks) and
- 4) the impact of osmolality changes in the reactor brought about by NaOH addition for pH control. The combined effects of CO_2 sparging and osmolality increases brought about by NaOH addition on cellular activities have been reviewed in the Introduction Chapter, Section 1.2.3. Inhibition of cell growth with increasing levels of dissolved CO_2 was noted in studies reported by deZengotita et al., (1998). A decrease in the growth rate of the cells by 86% in comparison to the growth rate observed for cells cultured in medium at an osmolality of 342 mOsm/kg. An important consideration is the adaptation of cells. When cells are transferred from one growth environment to another a period of adaptation is required. Another step further would therefore include efforts to adapt the cells to the reactor environment by conducting several passages of the cells in the minifors reactor.

The growth of cells in suspension will, however, become limited at a certain level. Various authors have reported on improved cell growth and enhanced recombinant protein yields through the manipulation of key culture parameters (including temperature, feeding strategy etc). This has however proven limited in successfully achieving increased cell and product yields with low specific productivities and maximum cells densities reported (1-10 $\text{pg}/\text{cell}/\text{h}$ and $1-5 \times 10^6$ cells/ml) as reviewed by Selimoglu, and Elibol, (2010). Therefore a more desirable approach is the investigation of the encapsulation of the cells in alginate-poly-L-lysine-alginate (APA). It is evident from this study that the cells are under stress in the bioreactor environment, therefore, alternatively efforts could be made to protect the cells from this environment as oppose to altering the conditions of the environment. The microcapsules would not only serve to protect the cells from their surrounding environment by entrapping them in a liquid core gel surrounded by a membrane of alginate cross-lined with poly-L-lysine, but it also provides a 3-D structure for the growth of mammalian cells, which have a natural tendency to aggregate. The cultivation of cells in close proximity to one

another enhances cell to cell communication for optimised cell growth and productivity. Immobilisation/entrapment/encapsulation of cells has been utilised as an effective tool for achieving higher cell densities than those which occur in optimal suspension conditions (Breguet et al., 2007; Peirone, 1998). However the outward effect on such a cultivation technique of recombinant protein quality is yet to be characterised.

Chapter 5: Encapsulation of CHO-DP12 cells in Alginate-Poly-l-lysine-Alginate microcapsules: Implications on rIgG1 quality

Abstract

A primary objective for the production of monoclonal antibodies on the industrial scale is sustained productivity at high cell densities. Various authors have reported on improved cell growth and enhanced recombinant protein yields through the manipulation of key culture parameters (including temperature, feeding strategy etc). Cell immobilisation has been suggested as a cultivation method which protects cells from bioreactor associated shear forces for sustained viability and enhanced productivity. The concentration of autocrine factors are considered to be locally higher inside the immobilisation matrix, as opposed to being diluted in the surrounding media when cells are present in suspension.

A study was designed which involved the encapsulation of a CHO DP-12 cell line, producing the recombinant protein IgG1 in Alginate-poly-L-lysine-alginate (APA) microcapsules. The aim of the experiment was to investigate increased cell and product yields than those achieved in previous suspension cultures. Encapsulated culture yields (cells/ml_{reactor}) were 3.7-fold higher than those achieved when cells are cultured in suspension. Due to the increased number of cells producing the recombinant protein, an increase in the volumetric productivity (4.8-fold) and final yield of rIgG1 was achieved in encapsulated cultures. The quality of the rIgG1 was also monitored and noted to be 99 % monomeric. The glycosylation profile of the rIgG1 were similar for both suspension and encapsulated cultures at the end of the stationary growth period. A greater increase in high mannose glycan structures was present over the stationary growth period in encapsulated cultures in comparison to suspension. It was attributed to the batch nature of the culture in which the concentration of glutamine was depleted by the end of the exponential phase, thus limiting the availability of glutamine derived nucleotide sugar precursors over the extended stationary phase which occurred in the encapsulated cultures in comparison to the suspension cultures.

Abbreviations

AFM	atomic force microscopy
APA	alginate-poly-L-lysine-alginate
CHO	Chinese hamster ovary
BHK	Baby hamster kidney

ER	Endoplasmic reticulum
FDA	Food and drinks authority
GA	golgi apparatus
GalNAc	N-acetylgalactosamine
GFAT	glutamine:fructose-6-phosphate
GlcNAc	N-acetylglucosamine
GU	glucose units
HEK	human embryo kidney
HEM-MMA	hydroxyethyl methacrylate-co-methyl methacrylate
IL-6	Interluekin-6
IgG	Immunoglobulin G
IL-6	Interluekin-6
IFN- α	Interferon- α
mAb	monoclonal antibody
MDCK	madin darby canine kidney
PLL	Poly-L-lysine
SEC	size exclusion chromatography
TGF β 1	Transforming growth factor β 1
UDP-GNAc	uridine di-phosphate N-acetylhexosamine
UTP	uridine tri-phosphate
VEGF _{165b}	Vascular endothelial growth factor165b

5.1 Introduction:

A primary objective for the production of monoclonal antibodies on the industrial scale is sustained productivity at high cell densities. Efforts have been made to improve maximum cell densities and increase recombinant protein yields through manipulation of environmental culture conditions. Biphasic approaches employing temperature shift optimisation allow for high cell densities to be achieved while inducing optimised specific productivities in the cells (Fox et al., 2004). Optimisation of media components and feed

strategies has alleviated the inhibition of cell growth and productivity associated with the accumulation of toxic by-products (Xie and Wang, 2006). Manipulation of culture conditions have however proven limited in successfully achieving increased cell and product yields with low specific productivities and maximum cells densities reported (1-10 pg/cell/h and $1-5 \times 10^6$ cells/ml) (Selimoglu, and Elibol, 2010). High cell densities are impeded by mechanical and shear forces resulting from agitation and aeration schemes in large scale industrial bioreactors. Cell immobilisation has been suggested as a cultivation method which protects cells for sustained viability and enhanced productivity (Seifert and Philips, 1999).

As stated in the Introduction Chapter, Section 1.2.7, microencapsulation was first used in the treatment of diabetes, in which pancreatic islet cells were first immobilised in alginate and delivered in vivo without the occurrence of a rejection reaction (Lin and Sun, 1980). Since then mammalian cell immobilisation has been used for various applications including the encapsulation of recombinant cells containing genes encoding therapeutic proteins, with enhanced cell densities and/or increased culture longevity being achieved, greater than those which occur for cells cultured freely in suspension (Sinacore et al., 1989; Lee and Palsson 1990; Ma et al., 2006; Breguet et al., 2007; Selimoglu et al., 2012). The advantages of encapsulation for mammalian cell cultivation leading to higher cell densities than those achieved in suspension have been described in the Introduction Chapter in Section 1.2.7, all of which relate to the provision of a protective environment for the fragile mammalian cells from shear forces associated with the bioreactor environment (Papoutsakis, 1991).

Initial studies performed by Lim and Moss (1981) suggested that a 10-fold increase in monoclonal antibody (mAb) yield could be achieved in immobilised hybridoma cultures in comparison to suspension cultures due to an increase in cell densities achieved, as reviewed by Selimoglu and Elibol, (2010). In relation to enhanced productivity associated with encapsulated cultivation of mammalian cells, the microcapsule environment is associated with enabling increased higher local concentrations of autocrine productivity promoting factors, such as Il-6 (Farrell et al., 1994). The microcapsule provides space limitation which limits cell proliferation. Once the higher cell densities have been achieved and the space within the microcapsules has been occupied, lower growth rates enable enhanced specific recombinant protein production (Yamaguchi et al., 1997). Enhanced productivity in immobilised culture platforms has been reported by various investigating authors to date (Sinacore et al., 1989; Lee and Palsson 1990; Lee et al., 1993; Peirone, 1998; Seifert and

Philips 1999; Rokstad et al., 2003 Breguet et al., 2007; Zhang et al., 2007; Selimoglu et al., 2012).

As reported in Chapter 4, the maximum viable cell density achieved for the Chinese hamster ovary (CHO) DP-12 cell line used, when cultured in a 1.7 L minifors reactor was $3.12 * 10^6$ cells/ml. The maximum concentration of recombinant immunoglobulin G (IgG1) produced in the culture was approximately 60 µg/ml. Previous shake flasks studies reported in Chapter 3 investigated the optimisation of cell growth and productivity through the manipulation of medium components, focusing on glutamine and ammonia concentrations. However the scaling up of these experiments, with optimised L-glutamine concentrations, did not enhance the cell or product yields achieved. Therefore in an effort to seek enhanced cell densities and product yields, encapsulation in alginate-poly-L-lysine-alginate (APA) microcapsules as a mode of cultivation was investigated for the CHO DP-12 cell line producing the recombinant protein IgG1. APA microcapsules are reportedly the most widely studied microcapsules applied to mammalian cell cultures for enhanced recombinant protein production (Zhang et al., 2007), due to a number of advantages associated with alginate, as outlined in the Introduction Chapter, Section 1.2.7. CHO cells were immobilised in a sodium alginate solution. Extrusion of the solution through a 300 µm diameter nozzle and the application of vibration nozzle technology allowed the formation of alginate gelled beads containing the cells, upon incubation in (0.1M) Ca_{2+} . Ca_{2+} is the most suitable ion in relation to biocompatibility of mammalian cells to the ion and also affinity for the sodium alginate. The beads were coated with PLL (Mw 30,000-75,000), followed by a second layer of alginate (0.03 % w/v) and liquefied with 0.05 M sodium citrate. The results of such liquefaction are that cells float freely around the interior of the capsule. (Gugerli, 2003). The APA microcapsules were used to inoculate the same 1.7 L minifors reactor in an approximate ratio of 300 ml of capsules and 900 ml of excel CHO 325 PF media. Batch cultures were performed in triplicate and the average seeding density was $0.32 * 10^6$ cells/ml_{reactor}, as deduced by initial cell counts performed immediately post inoculation.

The overall aim of the study was to investigate the implications of encapsulation as a mode of cultivation on the growth, metabolism and productivity of the mammalian cell line in culture in comparison to the profiles observed for the same cell line when cultured freely in suspension. Of the many studies, noted above, demonstrating the growth, recombinant protein production and stability of microcapsules in vitro (Breguet et al., 2007; Peirone, 1998; Rokstad et al., 2003), the effects, if any, such a mode of cultivation may have on the quality

of recombinant proteins produced (glycosylation and/or aggregation) have somewhat been overlooked. Enhanced high cell numbers and recombinant protein yields are only to be desired providing there is no trade-off in the quality of the protein produced. It may be hypothesised that due to the decreased growth rates associated with immobilised mammalian cells, the time in which recombinant proteins remain exposed to glycosylation enzymes may be increased. Enhanced volumetric productivity of the rIgG1 may promote aggregation through self association at high concentrations in the resulting culture media. However, the intrinsic structure of the APA microcapsule may provide a barrier to the release of the 150 kDa IgG molecules, promoting aggregation of the molecule inside the capsule. The reported molecular weight cut-off point of APA microcapsules is <100kDa. However in the presence of living cells, APA microcapsules have reportedly demonstrated full permeability to protein products with molecular weights ranging from 21-300kDa (Awrey et al., 1996). The diffusion of a solute through a membrane is not just dependent on the molecular weight cut-off of the membrane but also on the ionic charge, size, shape and radius of gyration of the solute (Tyn and Guesk, 1990).

5.2.Results:

To evaluate the effect of encapsulation as a mode of cultivation for mammalian cells, CHO DP-12 cells, which grow in suspension, producing the recombinant protein IgG1 were encapsulated in APA microcapsules. Approximately 300 ml of alginate containing $0.5 * 10^6$ cells/ml of alginate was extruded through a 300 μ m nozzle into a bath containing 110 mM CaCl₂ solution to form beads. The beads were then incubated for 30 minutes in poly-l-lysine. The core of the beads was liquefied through incubation in 0.05 M sodium citrate solution. The liquid core capsules were then used to inoculate a minifors reactor in a total culture volume of 1.2 L comprised of 900 ml of ExCell CHO 325 PF media, supplemented with 4 mM L-glutamine, and 300 ml of capsules.

Suspension cultures were also carried out in parallel. A total media volume of 1.2 L ExCell CHO 325 PF media, supplemented with 4 mM L-GLN was inoculated with CHO DP-12 cells producing the recombinant protein IgG1 at a cell density of $0.3 * 10^6$ cells/ml. Samples were taken on a daily basis for the determination of cell growth, metabolite concentration, IgG concentration, glycosylation and aggregation. Samples of microcapsules were also examined microscopically for size determinations. Microcapsule strength was determined every second

day using atomic force microscopy (AFM). Both encapsulated and suspension cultures were carried out in triplicate.

5.2.1. Characterisation of microcapsules

5.2.1.1. Capsule size

The determination of microcapsule size throughout the culture period is an important parameter as changes in the size affect the diffusion of metabolites and other cellular products in and out of the capsules. Large capsules (> 1 mm) may suffer diffusion limitations resulting in reduced cell growth and necrosis within the core. However it was reported that with greater bead sizes higher cell concentrations could be reached. Inoculum density is directly proportional to bead size in almost all encapsulation systems (Selimoglu and Elibol, 2010). The APA microcapsules were formed by the extrusion of a sodium alginate solution through a 300 µm nozzle. Due to liquefaction of the alginate core, swelling of such microcapsules in the range of 150-200 % of the original volume has been reported (Breguet et al., 2007). Based on previous studies, the final diameter of the microcapsules would be expected to be in the range of 700-900 µm. Capsule size determination was carried out using a laboratory microscope (Nikon Eclipse TI) with the corresponding software for image analysis (NIS-element AR). Thirty capsules were measured individually to calculate the average radius and standard deviation, as estimated by comparison with a calibrated scale. Generally size distribution was below 3 % between microcapsules in a given culture conditions at a given time.

Figure 5.2.1.1.1 below demonstrates the capsule radius size as determined from samples taken throughout the culture period in the triplicate cultures performed. The capsules size was determined microscopically. The average initial capsule radius for the 3 cultures performed was ~ 460 µm. The capsule size remained within a consistent range throughout the cultivation period. However it is important to note, that size was only determined for intact capsules. The appearance of broken capsules towards the end of the culture period, from day 7-9 was omitted. Capsule breakup by the end of the culture period, day 9, was determined to be 18 % of the total volume of capsules present.

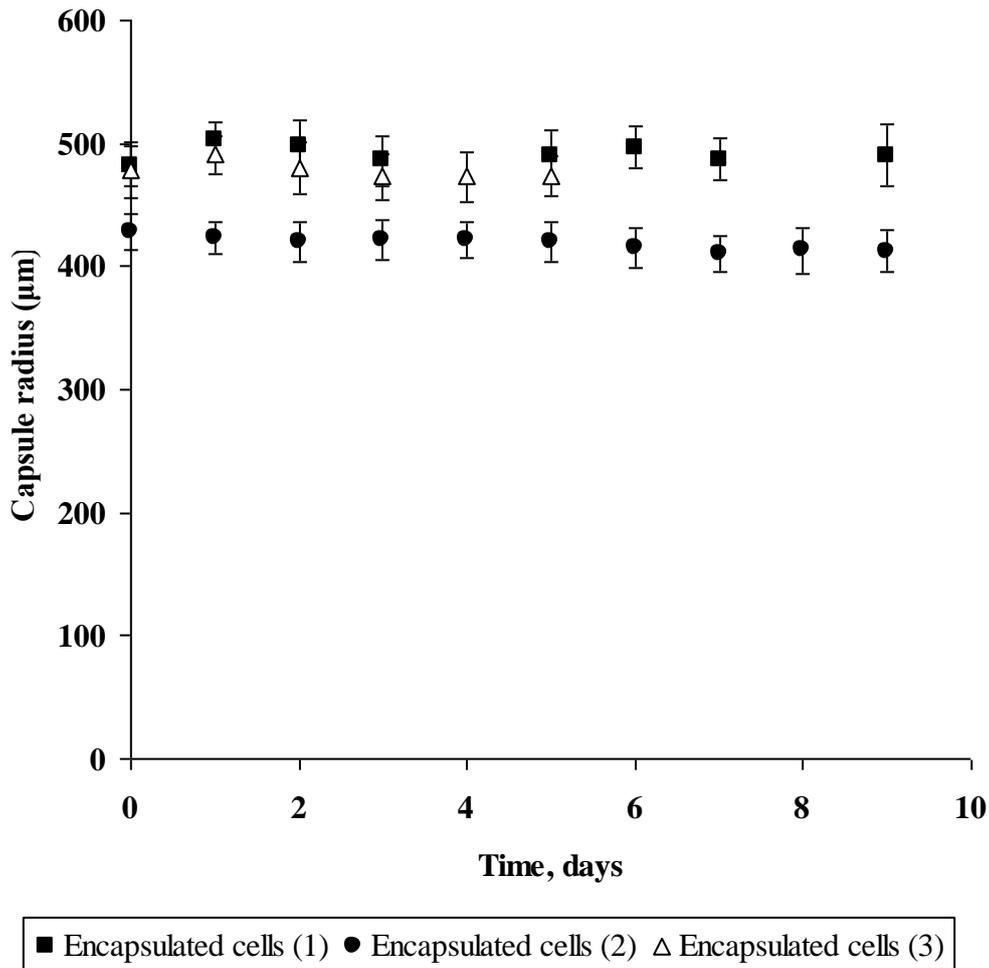


Figure 5.2.1.1.1: Capsule radius size (μm) over the duration of the cultivation period for each of the three encapsulated cultures completed. The size of approximately 30 capsules was measured at each time point. Values are the mean of 30 microcapsules analysed on a daily basis \pm STD.

5.2.1.2 Capsule strength

Capsule resistance is one of the critical factors when capsules are intended for bioreactor cultures. The bursting of capsules would result in the release of cells, cell components and genetic material. Furthermore, resistance is a key variable of microcapsules, being directly responsible for controlling the permeability (Gugerli, 2003). The mechanical strength of the bead is dependent upon the molecular size of the alginate used. Inflexibility in solution increases in the order of $\text{MG} < \text{MM} < \text{GG}$ content of the alginate (Selimoglu and Elibol, 2010). Samples of capsules from the cultures were evaluated every second day using a Texture Analyser.

Evaluation of Figure 5.2.1.2.1 below indicates a decrease in the force (g)/capsules over cultivation time, representative of a decrease in the mechanical resistance of the APA microcapsules throughout the culture period. The decrease in capsule strength mainly occurred during days 0-3 (inclusive) in which the force (g)/capsule decreased from 0.49 +/- 0.09 g/capsule to 0.07 +/- 0.01 g/capsule. The decrease in microcapsule strength during this time period may be attributed to the introduction of shear forces to do the capsules in the reactor and also an equilibration with the medium. As no reactor conditions changed in this batch culture over time, no further decrease in capsule strength was noted later in the culture period. However the integrity of these weakened capsules became an issue from day 7 onwards when there was an estimated 18 % loss of capsules due to breakage in the culture. This may be due the fact that these weakened capsules then contained the maximum viable cell number observed in the culture (day 6). The method used to determine the force (g)/capsule analysed a layer of capsules at a time. This method is effective for efficient measurement of large numbers of capsules. However the results are affected by large size distributions. It was however noted in Figure 5.2.1.1.1 that the size distributed between capsules is typically low, 3-5 %.

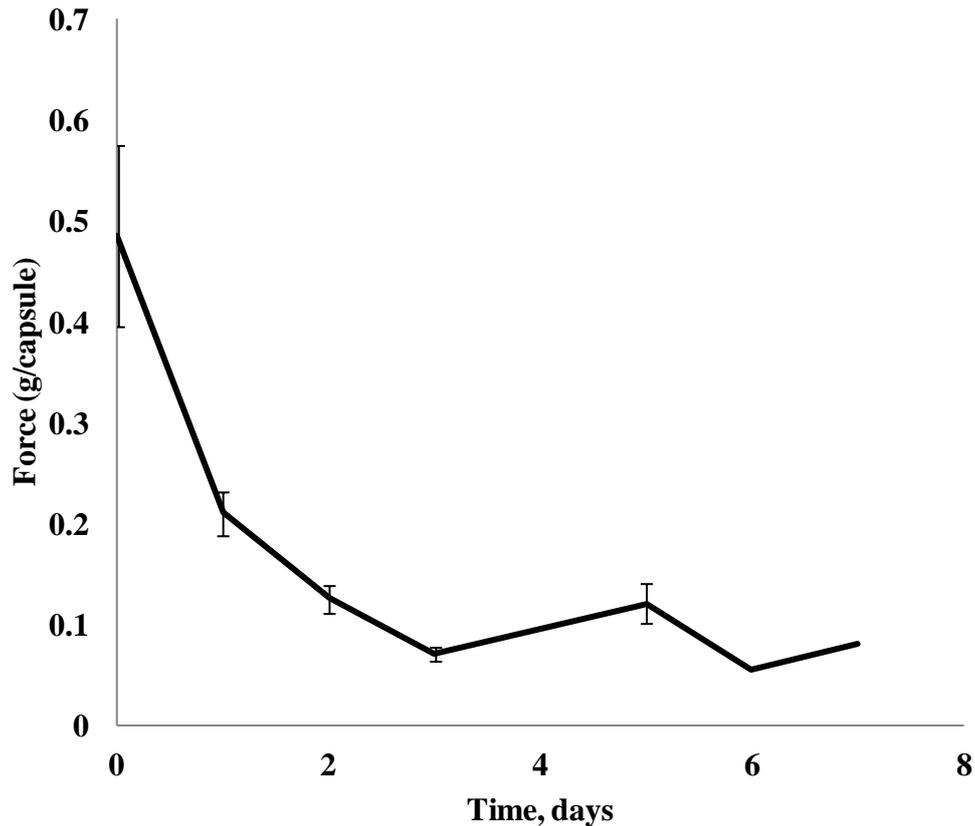


Figure 5.2.1.2.1: Mechanical resistance (force (g)/capsule) over cultivation period for 300 μm radius APA microcapsules containing CHO DP-12 cells cultivated in a 1.7 L minifors reactor. A total of 3 layers of microcapsules were analysed on a daily basis. Values presented are average of such triplicate analysis \pm STD for one of the encapsulated cultures carried out in the study.

5.2.1.3: Permeability of microcapsules

The permeability of the microcapsules to dextrans ranging in molecular weights, up to 500 kDa and including the molecular weight of the IgG1 protein which the cells produce (150 kDa), was determined for the 300 μm radius APA microcapsules. 300 μl of capsule suspension was incubated in 600 μl of a 0.05 % fluorescent dextran solution of a particular molecular weight. The capsules were incubated under agitation for 2 hours and analysed using confocal microscopy.

Figure 5.2.1.3.1 below illustrates the permeability of the microcapsules used in these cultures to the fluorescent dextrans of molecular weight 150 and 500 kDa. The method was not optimised at this point for quantitative determination of the molecular weight cut-off of the

microcapsules. However such image analysis which was performed on days does indicate that the microcapsules were permeable to components of molecular weights > 150 kDa. The IgG1 protein produced by the cells is 150 kDa.

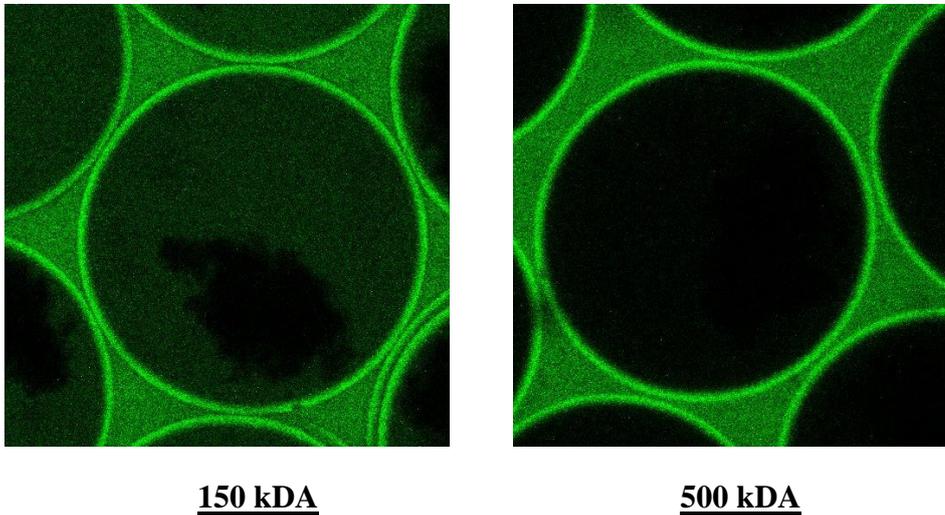


Figure 5.2.1.3.1: Confocal microscopy analysis (100 X) of 300 μm APA microcapsules incubated in fluorescent dextran (150-500 kDa) at a volume ration of 1:3 microcapsules: dextran solution

5.2.2.: Effect of encapsulation on CHO cell growth

5.2.2.1 Growth of encapsulated cells

Microscopic analysis on a daily basis of the microcapsules enabled direct visualisation of the colonisation of the microcapsules with cells. 300 ml of alginate was inoculated with a cell density of $0.5 * 10^6$ cells/ml alginate. Prior to such an inoculation the required cell number was calculated and the appropriate volume of a suspension culture was centrifuged at 200 g for 5 minutes. The resulting pellet was resuspended in the alginate using gently aspiration. Appropriate mixing, by means of aspiration was essential at this point in order to ensure sufficient distribution of the cells in the alginate microcapsules. Figure 5.2.2.1.1 below illustrates the colonisation of the cells in the microcapsules over the cultivation period (days 0, 2, 4 and 6). Examination of day 0 illustrations indicates that the inoculum was evenly distributed throughout the capsules formed. Very few empty capsules were evident. However, irrelevant of the even distribution of cells throughout the capsules on day 0, the cells appear to grow in extremely close proximity inside the capsules, forming one distinct cluster of cells in each capsule. Animal cells have a natural tendency to aggregate;

therefore an immobilisation process will offer optimal growth conditions. Figure 5.2.2.1.2 below illustrates the morphology of the cells in the clusters on day 6 of the cultivation period. The cells do not appear to be aggregated, but conform to their single cell morphology common of their growth in suspension.

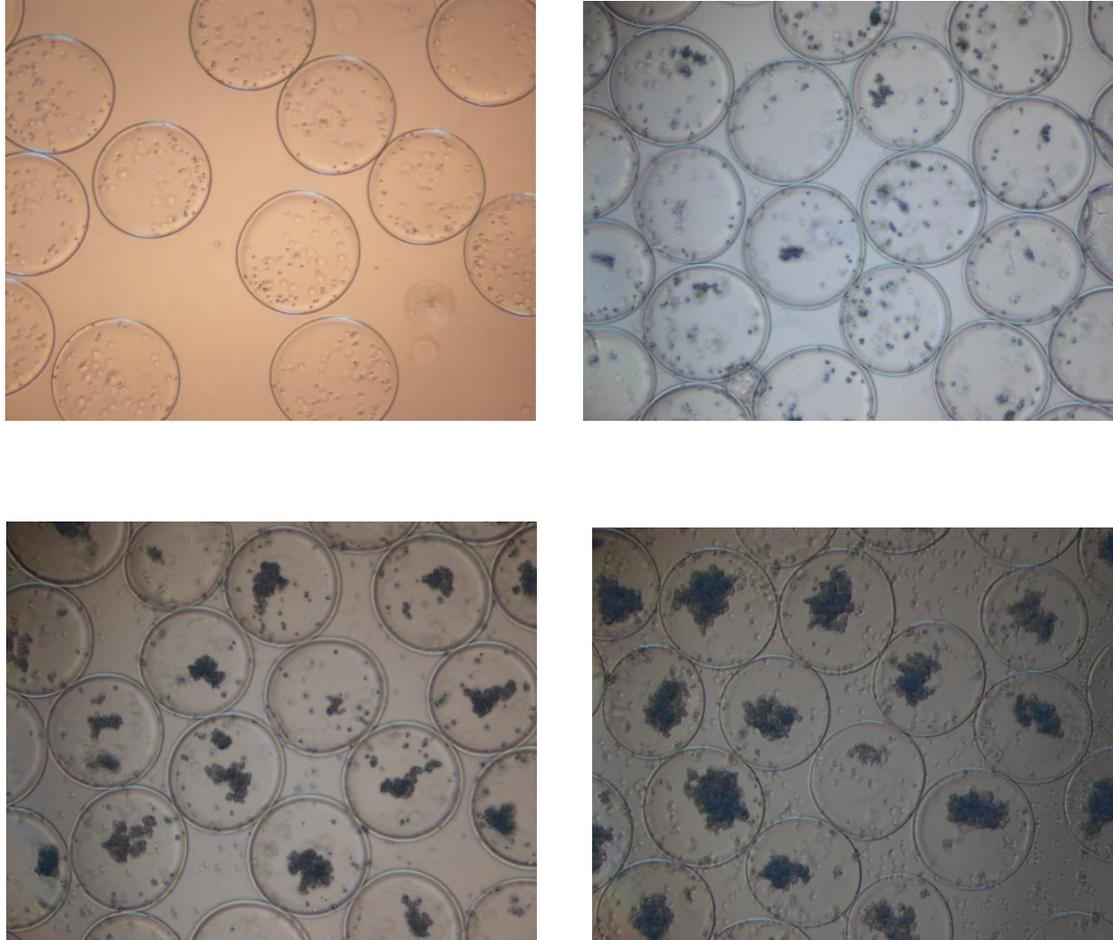


Figure 5.2.2.1.1: Microscopic analysis (40 X) (unstained) of microcapsule colonisation by cells over the cultivation days 0, 2, 4 and 6. Cells were originally seeded at 0.5×10^6 cells/ml alginate and which was extruded through a 300 μm nozzle as part of the liquid core microcapsule formation. The resulting microcapsules were used to seed a reactor with a ratio of 300 ml capsules and 900 ml media.

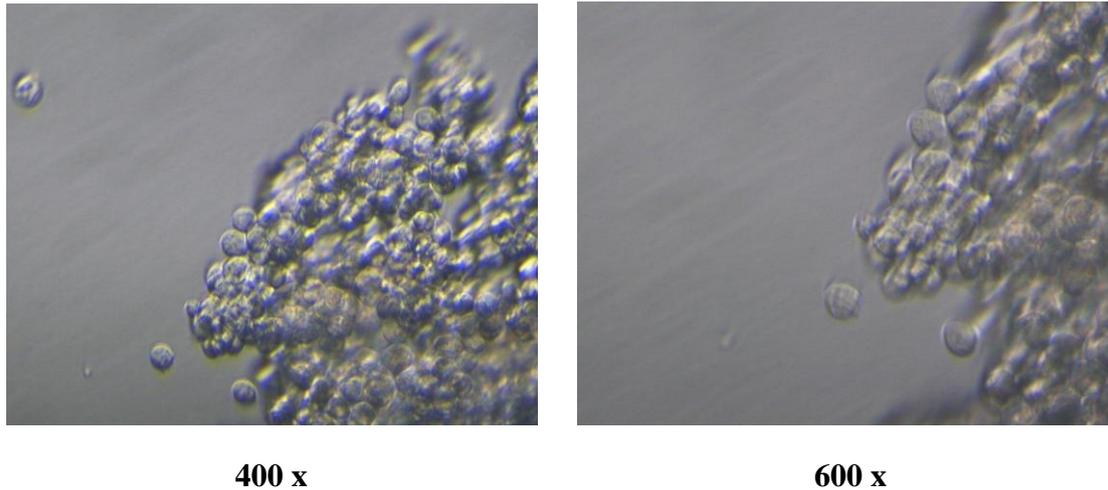


Figure 5.2.2.1.2: Microscopic analysis (400 x and 600 x) (unstained) of the cluster of cells present inside microcapsules on day 6 of the culture period. Microcapsules were originally seed at $0.5 * 10^6$ cells/ml alginate. Although cells are evenly distributed throughout the capsules on day 0, growth patterns displayed by the cells demonstrate the formation of clusters of singly growing cells inside the capsules.

5.2.2.2 Growth of cells in capsules -v- suspension

Microcapsules formed through the extrusion of 300 ml of alginate inoculated with $0.5 * 10^6$ cells/ml_{alginate} through the 300 μ m nozzle were used to inoculate a minifors reactor containing 900 ml of media. Due to variations in the encapsulation procedure the average seeding viable cell density of the three encapsulated cultures performed was $0.32 * 10^6$ cells/ml_{reactor} +/- $0.165 * 10^6$ cells/ml. The average seeding density of the three suspension cultures inoculated in 1.2 L of media in the minifors reactor was $0.30 * 10^6$ cells/ml_{reactor}. All bioreactor conditions remained the same between the two modes of cultivation. To evaluate the effect of cell encapsulation on the growth of cells, samples were removed from the reactor on a daily basis. Cell counts were performed using the trypan blue exclusion method and haemocytometer. Prior to performing cell counts, microcapsules were ruptured by extrusion through a 300 μ m needle and diluted in PBS.

Figures 5.2.2.2.1 and 5.2.2.2.2 and the growth stats presented in Table 5.2.2.2.1 below, allow the growth patterns and viability of the cells in the various cultures to be compared and contrasted. The maximum specific growth rate displayed by the cells in suspension and encapsulated in 300 μ m radius capsules was similar. Both culture conditions resulted in a lag period of 1 day before exponential growth was achieved. Encapsulated cultures spent approximately 2 extra days in exponential phase resulting in a maximal viable cell density 3.7

fold higher than that achieved when the cells were cultured in suspension. A short stationary phase of 1 day existed in the growth profiles displayed by cells cultured in suspension. A stationary phase of 2 days was observed for cells cultured in the liquid capsules. This is supported by the sharp fall off in viability one day after the maximum viable cell density has been achieved, day 5 for suspension cultures and day 7 for encapsulated cultures. The initial viability of the encapsulated cells was slightly lower than that displayed in the suspension cultures. However during exponential growth it was maintained above 85 %.

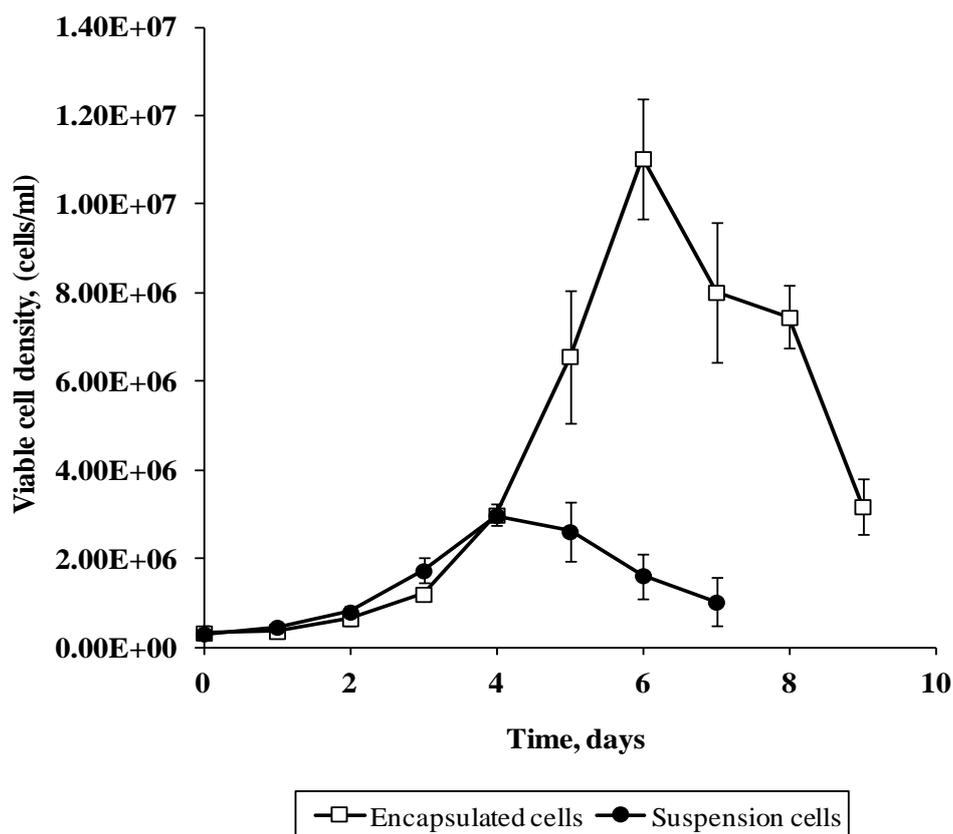


Figure 5.2.2.2.1: Growth of CHO cell cultures in suspension and encapsulated 300 μm radius liquid core APA microcapsules. The cell concentration is given with respect to the volume of the reactor and not with respect to the capsule's volume. Encapsulated and suspension cultures were seeded at an average viable cell density of 0.3×10^6 cells/ml_{reactor}. Cell numbers were enumerated on a daily basis using trypan blue exclusion method and a Neubauer haemocytometer. Encapsulated cells were liberated prior to counting via extrusion of a capsule/PBS suspension through a 300 μm needle 3 times. Duplicate cell counts were performed. Values presented above are the average of triplicate cultures carried out in parallel +/- STD, for both culture conditions tested.

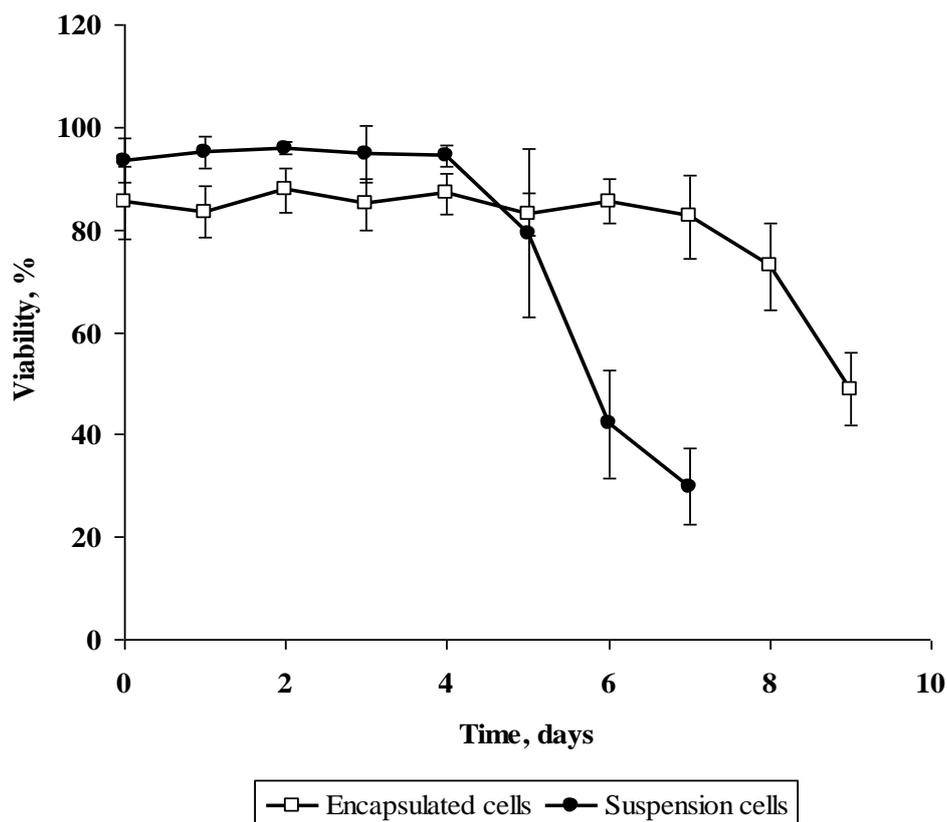


Figure 5.2.2.2.2: % Viability of CHO cells cultured in suspension and encapsulated in 300 μm liquid capsules. % viability was determined by the enumeration of total and live cell numbers present in the culture using trypan blue exclusion method and Neubauer haemocytometer. % viability is expressed as a percentage of live cells over dead cells present. Cell counts were performed in duplicate and average % viability determined on a daily basis. Values presented above are the average of triplicate cultures carried out in parallel \pm STD.

Table 5.2.2.2.1: Calculated maximum specific growth rates displayed by cells cultured in suspension and encapsulated in 300 μm liquid capsules over the exponential growth period. The growth rate was calculated from a plot of the logarithm of the viable cell density over the cultivation time (days). The maximum viable cell density is presented with respect to per ml reactor working volume. The results presented below are the average determined for triplicate cultures performed for each of the two conditions tested.

Culture	μ_{max} (day^{-1})	Max VCD ($\ast 10^6$ cells/ml)
Encapsulated cells	0.759 \pm 0.12	11.02 \pm 1.35
Suspension cells	0.645 \pm 0.0377	3.12 \pm 0.273

5.2.3: Effect of encapsulation on rIgG1 production

To evaluate the effect of the two modes of cultivation on rIgG1 production, media samples were removed from the suspension cultures on a daily basis from day 4 onwards and on day 5 onwards from the encapsulated cultures. As explained in Section 2.3.6., of the Materials and Methods Section, rIgG1 was purified from media samples removed from the cultures after centrifugation at 200 g for 5 minutes to remove cells. Capsules were separated from the media before the centrifugation step for the encapsulated culture samples. rIgG1 present in the media was purified using Protein A Nab spin columns and quantified using the nanodrop at A 280 nm.

Figure 5.2.3.1 below allows comparison of the concentration of rIgG1 produced by cells cultivated in both suspension and encapsulated in 300 μm liquid capsules. The values presented are the mean of triplicate cultures \pm standard deviation. The concentration of IgG1 produced by the encapsulated cells was not outside the standard deviation of the concentration produced by the cells cultured in suspension. This is due to the fact that the specific production rate displayed by the encapsulated cells was similar to that displayed by the cells cultured in suspension. Overall an increase production rate was noted for the encapsulated culture, as noted in Table 5.2.3.1. This was however due to the increased number of cells present at each time point and also the ended cultivation period. As noted in Figure 5.2.3.2, an increased concentration of rIgG1 was harvested from the encapsulated cultures at the end of the exponential and stationary growth phases than that which was harvested from the suspension cultures for the same culture stages. A greater increase in the concentration of rIgG1 was measured at the end of the stationary phase in comparison to the end of the exponential growth phase in the encapsulated cultures. This observation is due to the increased cell number producing the protein. At the end of stationary phase the concentration of rIgG1 in encapsulated cultures was an average of ~ 3.1 - fold higher than that observed in suspension cultures. This 3.1- fold increase is in line with the 3.7- fold increase noted in viable cell number and therefore verifying that there was no changes in the specific production rate.

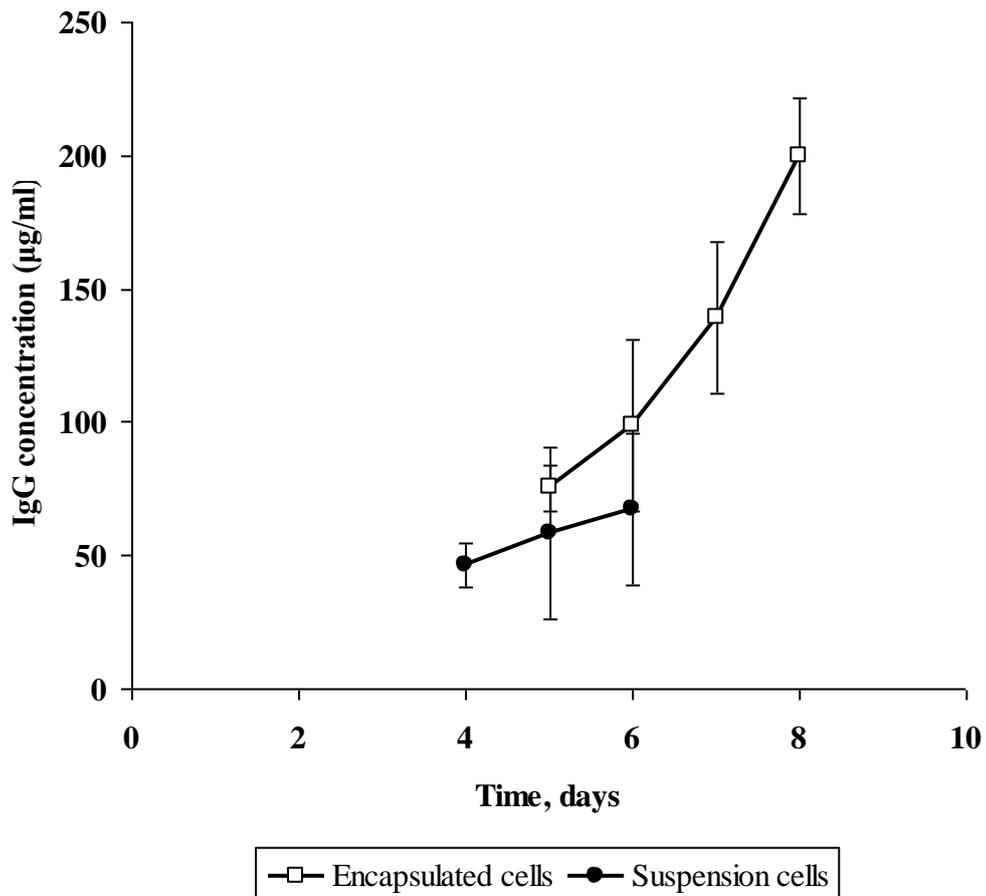


Figure 5.2.3.1: Concentration of rIgG1 purified from cell free media samples harvested from encapsulated cultures on day 5 onwards and suspension cultures on day 4 onwards. rIgG1 present in the media was purified using commercially available protein A/G Nab spin columns and quantified using the nanodrop A280 nm in duplicate which the average value recorded. Values presented above are the average of triplicate cultures, +/- STD, carried out in parallel for both culture platforms.

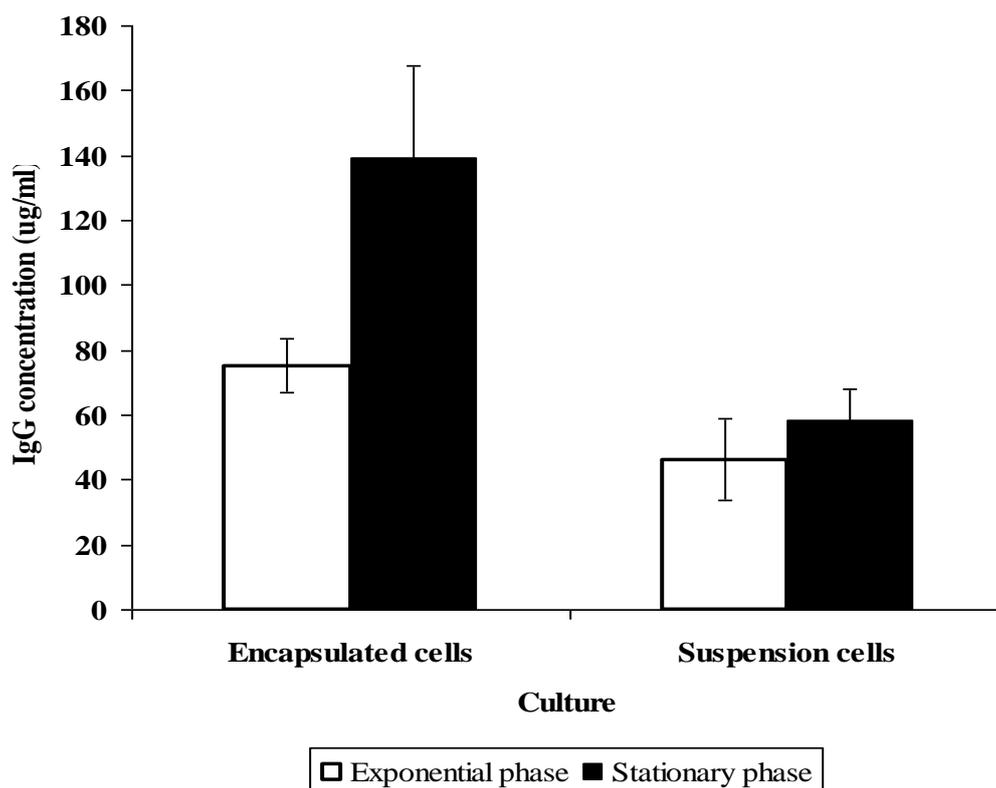


Figure 5.2.3.2: Concentration of rIgG1 purified from cell free media samples harvested from the encapsulated and suspension cultures at the end of the exponential and stationary growth period. rIgG1 was purified using Protein A/G spin columns and quantified using nanodrop A280 nm. Samples were quantified in duplicate with the average value recorded. Values presented above are the average of triplicate cultures carried out in parallel +/- STD.

Table 5.2.3.1: Calculated rIgG1 production rates and cell specific production rates for cells cultured in suspension and encapsulated in 300 μm radius liquid capsules. The specific production rate was calculated over the exponential growth phase for all cultures. The results presented below are the average of triplicate cultures, +/- STD, carried out in parallel for the two culture conditions tested

Culture	IgG1 production rate $\mu\text{g/ml/day}$	q_{IgG1} $\mu\text{g}/10^6$ cells/day
Suspension cells	10.52 +/- 2.80	3.74 +/- 0.23
Encapsulated cells	50.74 +/- 5.38	2.39 +/- 1.02

5.2.4 Effect of encapsulation on cell metabolism

Both encapsulated and suspension cultures were performed in ExCell CHO 325 PF media supplemented with 4 mM l-glutamine. The concentration of glucose in the commercially available media is expected to be 17.4 mM. The concentration of the major carbon sources, glutamine and glucose, and their corresponding by-products, ammonia and lactate, were measured from media samples taken from all cultures on a daily basis (Figure 5.2.4.1 and 5.2.4.2). Samples were removed from cultures on a daily basis and centrifuged at 200 g for 5 minutes, followed by filtration of the supernatant (0.22 µm PTFE) in order to remove cells and culture debris. Glucose and lactate concentrations were determined using HPLC analysis, and glutamine and ammonia analysis was carried out using commercially available enzymatic assays kits, as described in Materials and Methods Section 2.3.3. Determination of the concentration of the various metabolites enabled the calculations of the specific substrate uptake rate for each of the experimental cultures (Table 5.2.4.1).

An evaluation of the consumption profiles of glucose for both suspension and encapsulated cultures suggests that the different modes of cultivation impacted on the utilisation of this carbon energy source by the cells. In suspension cultures, all of the glucose was consumed at the end of the exponential growth phase. However, for cells cultured in 300 µm radius liquid capsules, glucose was not depleted until the end of the stationary growth period. Both the rate of glucose consumption and the cell specific glucose consumption rate were 60 % and 80 % higher, respectively, in the suspension cultures in comparison to the encapsulated cultures. As a result of increased glucose consumption a 3-fold higher concentration of lactate was accumulated in the suspension cultures in comparison to the encapsulated cultures. A 50 % higher yield of lactate on glucose occurred over the culture period in the suspension cultures in comparison to the encapsulated cultures.

The second major carbon source in mammalian cell culture, glutamine, was depleted at the end of the exponential phase in encapsulated cultures. It was not completely depleted until the end of the stationary phase in the suspension cultures. However there was a 73 % decrease in the cell specific consumption rate of glutamine in encapsulated cultures in comparison to suspension cultures, suggesting that glutamine was utilised more efficiently by cells cultures in liquid capsules, as oppose to in suspension. The decreased cell specific glutamine consumption rate noted in encapsulated cultures is coupled with a decreased cell specific ammonia production rate in the encapsulated cultures in comparison to the suspension cultures. As both the cell specific consumption and production rates decreased

proportionally in the encapsulated cultures in comparison to the suspension cultures, there was no significant difference in the yield of ammonia on glutamine between the two culture conditions. The yields were calculated for each of the three cultures carried out in parallel for the two conditions tested in this study. The results presented in Table 5.2.4.1 are the average of the triplicate cultures performed for each condition tested. As noted there was a high standard deviation for the yield of ammonia on glutamine for the three cultures performed for each condition tested.

The results may suggest an alternative growth limiting substrate between the two modes of cultivation (glucose for suspension cultures, meanwhile glutamine for encapsulated cultures). However it must also be considered that the high concentration of lactate may have also been responsible for the rapid decline in viable cell density noted in the suspension cultures.

It must be noted that there is a difference in the initial concentration of the two carbon energy sources between the suspension and encapsulated cultures. This is due to a dilution of the encapsulated cultures due to the ratio of capsules to media.

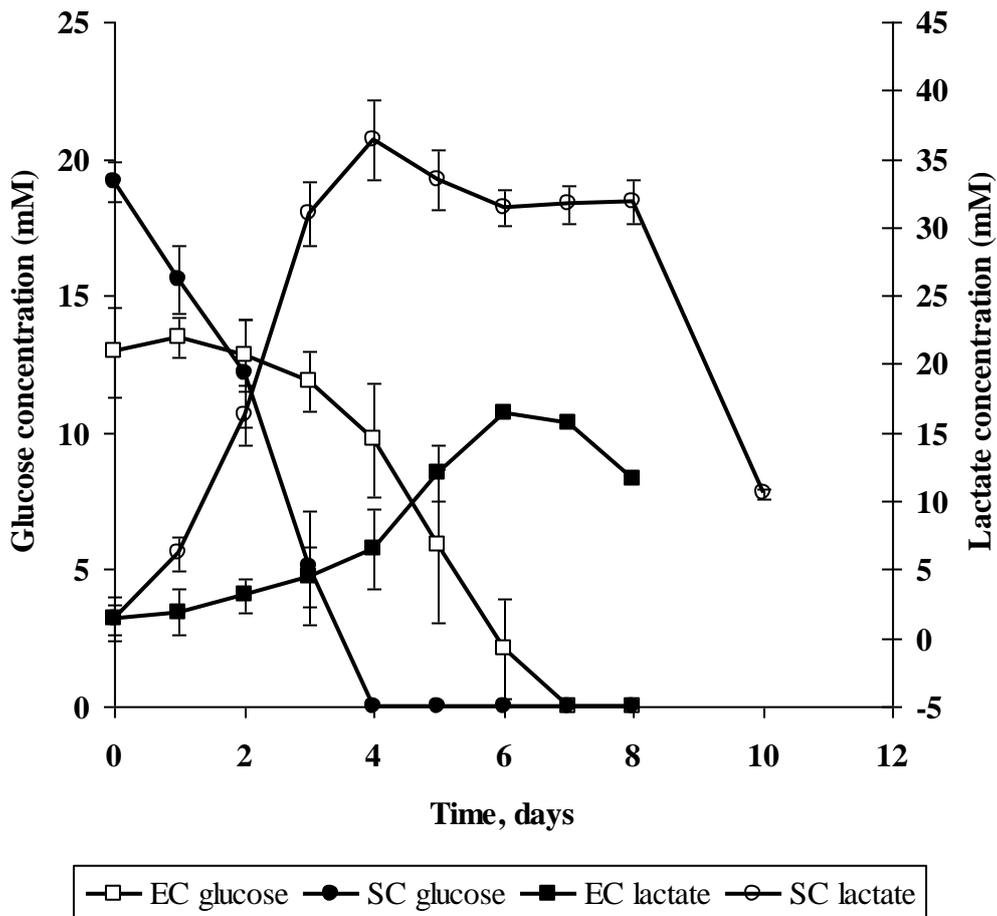


Figure 5.2.4.1: Concentration of glucose and lactate present in both suspension cultures (SC) and encapsulated cultures (EC) over the duration of the respective cultivation periods. The concentrations of glucose and lactate were determined offline by HPLC analysis using Supelco H column. Values presented above are the average of triplicate cultures carried out in parallel, +/- STD, for each of the two conditions tested.

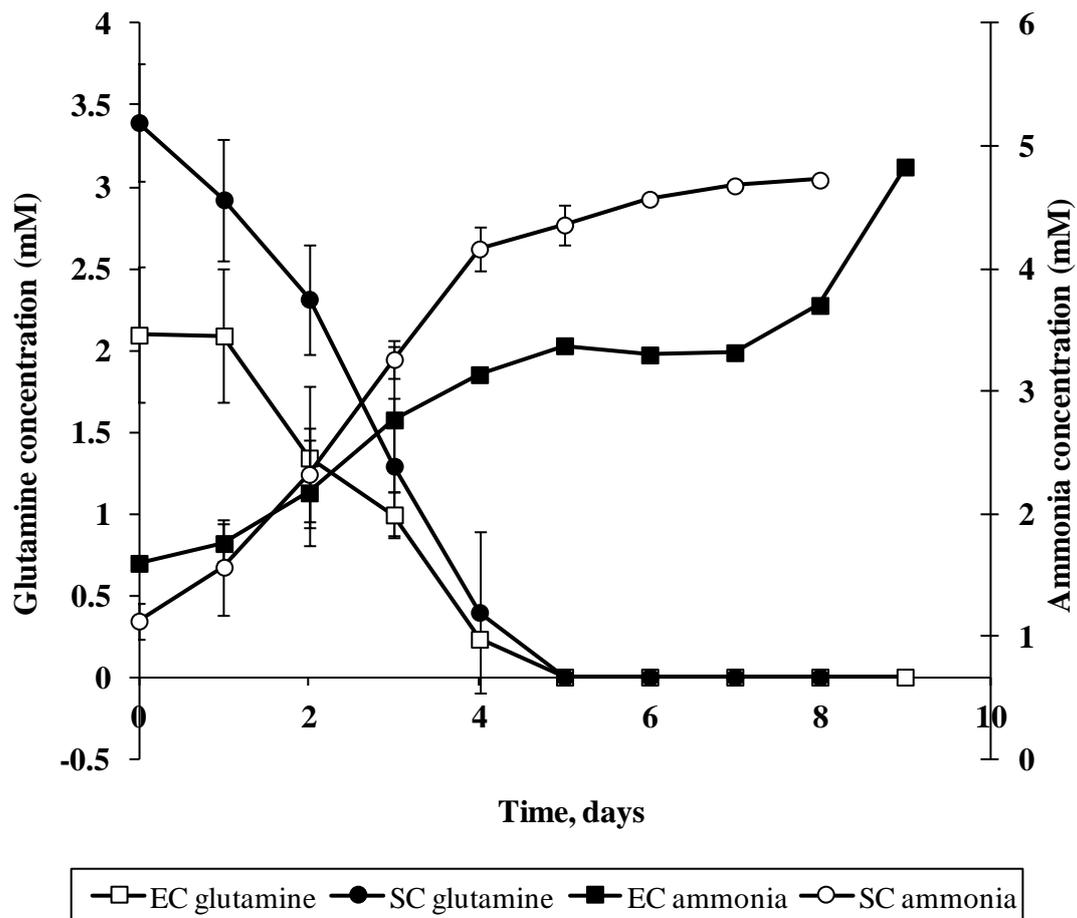


Figure 5.2.4.2: Concentration of glutamine and ammonia present in both suspension cultures (SC) and encapsulated cultures (EC) over the duration of the respective cultivation periods. The concentrations of glutamine and ammonia were determined offline using a commercial available enzymatic assay kit. Values presented above are the average of triplicate cultures carried out in parallel, +/- STD, for each of the two conditions tested.

Table: 5.2.4.1: Calculated rates of consumption and production of key metabolites as determined by offline analysis throughout the culture period. Cell specific consumption and production rates of the key metabolites were determined during the exponential growth phase. Yields of by-product on metabolite were calculated over the cultivation period. The results presented below are the average of that calculated for triplicate cultures carried out in parallel for each of the culture conditions tested.

	Encapsulated cells	Suspension cells
Glucose consumption rate (mM/day)	2.09 +/- 0.12	5.37 +/- 0.39
Glutamine consumption rate (mM/day)	0.59 +/- 0.19	0.81 +/- 0.057
Lactate production rate (mM/day)	2.55 +/- 0.71	10.54 +/- 0.40
Ammonia production rate (mM/day)	0.459 +/- 0.18	0.78 +/- 0.04
q_{glucose} (mmoles/ 10^6 cell/day)	$3.11 * 10^{-4} +/- 1.12 * 10^{-4}$	$1.69 * 10^{-3} +/- 1.00 * 10^{-4}$
$q_{\text{glutamine}}$ (mmoles/ 10^6 cell/day)	$8.42 * 10^{-5} +/- 6.21 * 10^{-6}$	$3.00 * 10^{-4} +/- 3.31 * 10^{-5}$
q_{lactate} (mmoles/ 10^6 cell/day)	$5.32 * 10^{-4} +/- 1.88 * 10^{-4}$	$3.07 * 10^{-3} +/- 3.3 * 10^{-4}$
q_{ammonia} (mmoles/ 10^6 cells/day)	$1.31 * 10^{-4} +/- 1.16 * 10^{-5}$	$3.46 * 10^{-4} +/- 2.74 * 10^{-5}$
$Y_{\text{lactate/glucose}}$ (mmol/mmol)	1.22 +/- 0.18	1.81 +/- 0.09
$Y_{\text{ammonia/glutamine}}$ (mmol/mmol)	1.88 +/- 0.23	1.23 +/- 0.15

5.2.5: Effect of encapsulation of on product quality

To assess the affect of encapsulation as a mode of cultivation for suspension cells on the quality of rIgG1 produced, both glycan and aggregation analysis were completed on the purified rIgG1 samples harvested from both the encapsulated and suspension cultures at both

the end of the exponential and stationary growth phases To study the influence of encapsulation as a mode of cultivation on the glycosylation of rIgG1, samples harvested from the suspension and encapsulated cultures were subjected to N-glycan release and labelled as outline in Materials and Methods Section 2.3.7.1. HILIC characterisation of the released and labelled N-glycans produced a chromatogram consisting of 8 distinctly separated peaks. Each of the peaks was assigned a distinctive glucose units (GU) value by comparison to the retentions times of the standard A-AB labelled external dextran. Examination of exoglycosidase treated samples allowed for the corresponding number and linkage of the component monosaccharide units represented by each GU value to be elucidated, as described in Materials and Methods section 2.3.7.3. Aggregation analysis of the purified samples was carried out using size exclusion chromatography (SEC)

Figure 5.2.5.1 below illustrates the relative % of the different glycans present on rIgG1 samples harvested from the cultures at the end of the stationary growth phase.

The earliest form of glycosylation, and an intermediate in the formation of more complex glycans is M5. There was a 25 % decrease in the relative present of M5 glycan structures present on rIgG1 samples harvested from encapsulated cultures, in comparison to those harvested from suspension cultures at the end of the exponential growth period. By the end of the stationary growth phase, the relative % of M5 on IgG1 protein harvested from both cultures had increased from the amount present on the protein at the end of the exponential growth phase. The increase in M5 over the stationary phase for suspension cultures was not significant. There was however a ~2.8- fold increase in M5 present on the protein harvested from encapsulated cultures at the end of the stationary phase, compared to that harvested at the end of the exponential phase. The increase in M5 in encapsulated cultures was much more dramatic than that observed in suspension cultures. Therefore although encapsulation did initially seem to be beneficial in resulting in reduced levels of M5 at the end of the exponential phase, there was in actual fact no significant difference in the glycoform profile of the protein harvested from both culture conditions by the end of the stationary growth period. These studies do suggest that should the stationary phase of the encapsulated cultures be lengthened, a lower quality product could result due to the increasing levels of non-complex M5 noted to occur for encapsulated cultures over this time frame. As there were no significant decreases in the relative % of the more complex glycoforms, the increase in the levels of M5 over the cultivation period cannot be attributed to the action of proteases released from the cells. In such cases increased occurrence of M5 is associated with a

depletion of nutrient over time which act as nucleotide sugar precursors, as is explained in detail in Discussion section 5.3.

Figure 5.2.5.2 illustrates the % of rIgG1 purified from cultures in a monomeric form. Over 95 % of the rIgG1 harvested from culture media was in a monomeric form. Similarly IgG1 harvested from suspension cultures demonstrated to be 99 % in a monomeric form.

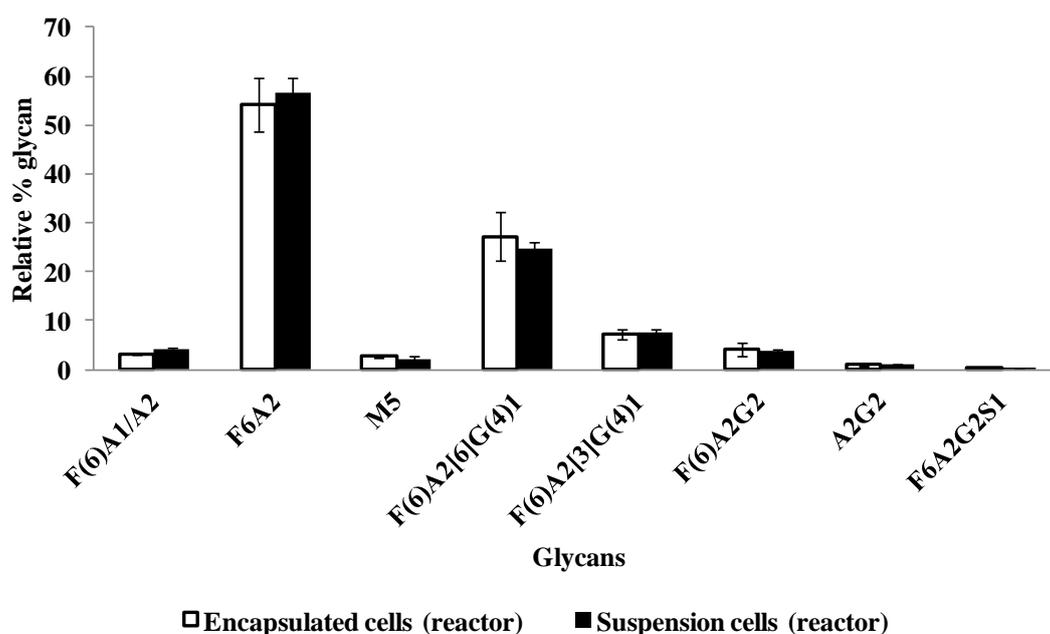


Figure 5.2.5.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from 1 L encapsulated and suspension cultures at the end of the stationary growth phase. N-glycans were released from the rIgG1 samples by means of PNGaseF digestion. Samples for HILIC analysis were labeled by adding 5 μ l of 2AB labelling solution. The retention time of the glycan peaks was compared with an external A-AB labelled dextran standard for structural elucidation by converting the retention time of the peaks into GU. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for each condition tested, +/- STD.

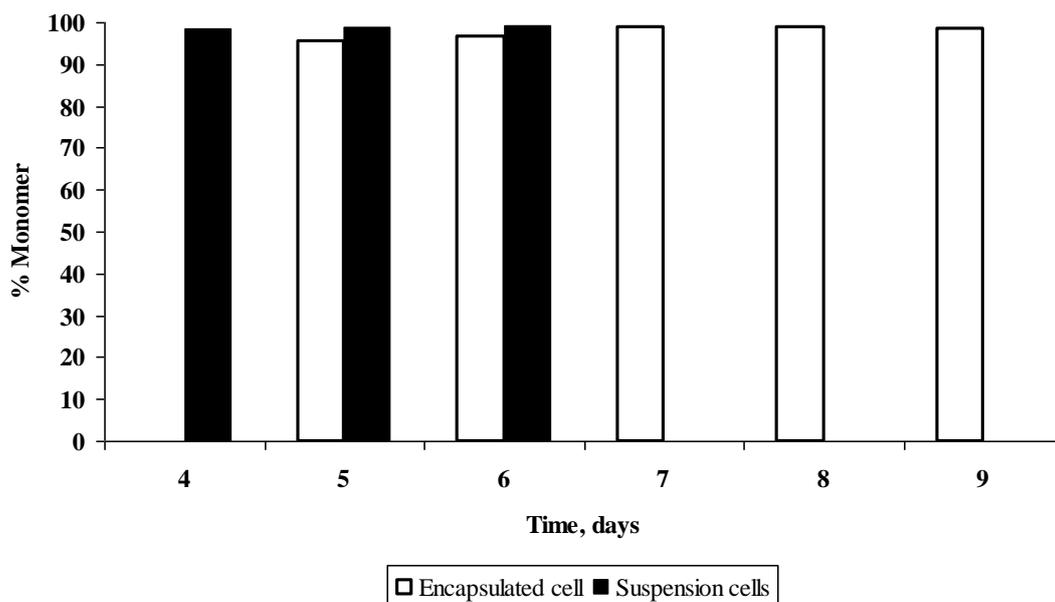


Figure 5.2.5.2: Relative % monomeric from of rIgG1 samples as detected by SEC. IgG1 samples were harvested from encapsulated and suspension cultures performed. IgG1 samples were analysed in duplicate. The results are presented as the average of triplicate cultures performed for the two culture conditions tested, +/- STD.

5.3: Discussion

The beneficial effects of cell immobilisation in 3-D microcapsules have been extensively reviewed since the first encapsulation of pancreatic islet cells for the treatment of diabetes (Selimoglu, and Elibol, 2010). The application of encapsulation to recombinant cell lines producing therapeutic proteins both in vivo (Liu et al., 1993; Change et al., 1993; Hughes et al., 1994.) and in vitro, as reviewed by Selimoglu, and Elibol, (2010) has been extensively documented with increased cell yields and concentrations of recombinant proteins produced coming to the fore. Results have been however been varied with cell line and cell type. Lee et al., (1994) tested a number of hybridoma cell lines and noted that increased specific mAb productivities demonstrated when cells are immobilised is cell line specific. There has also been a lack of research conducted regarding the implications of encapsulation as a mode of cultivation on recombinant protein quality. A study was therefore conducted which investigated the effects of CHO DP-12 cultivation in APA microcapsules on cell growth, metabolism rIgG1 production and also the quality (glycosylation and/or aggregation state) of the produced rIgG1. APA microcapsules containing the CHO cells were

used to inoculate a 1.7 L minifors reactor at an average initial cell density of $0.32 * 10^6$ cells/ml_{reactor} with a ration of 300 ml capsules and 900 ml of media. Free suspension cultures of the CHO DP-12 cells were carried out in parallel with the same initial seeding density of $0.30 * 10^6$ cells/ml_{reactor}.

The growth profiles of cells encapsulated in 300 µm radius microcapsules were characterised by both microscopic analysis of the microcapsules on a daily basis and also the performance of cell counts using a haemocytometer and trypan blue exclusion method, proceeding rupturing of the capsules. Cells were initially seeded in a homogeneous manner, evenly distributed throughout the microcapsule core. Growth of the cells resulted in the formation of cell clusters within the capsule core. The vast majority of research conducted on the growth of mammalian cells in microcapsules in vitro reported consistently similar results (Peirone et al., 1998; Afkhami et al., 2012; Bohari et al., 2011; Ma et al., 2006; Afkhami et al., 2010). It is common that cells, which are already adherent cell lines, would form cell-to-cell associations for viability when placed in a microenvironment. Lahooti and Sefton, (2000) reported the formation of single aggregates from free floating adherent human embryo kidney (HEK) cells in the core of poly(hydroxyethyl methacrylate-co-methyl methacrylate (HEM-MMA) capsules, just one day post-encapsulation. The aggregation of suspension adapted cell lines, such as CHO, has also been reported extensively in encapsulated cultures. Zhang et al., (2007) reported on the formation of small spherical aggregates of CHO cells during first 4 days post-encapsulation in APA microcapsules, which rapidly enlarged throughout the cultivation period. The morphology of cells inside microcapsules does appear to be dependent upon the seeding density used. Wen-tao et al., (2005) reported that for cells seeded at $5 * 10^5$ cells/ml_{capsules}, cells formed very few light aggregates. However at a seeding density of $5 * 10^6$ cells/ml_{capsules}, cells grew to form multiple very large aggregates. It was concluded that as cells altered from single cell morphology initially to an aggregated state inside the capsules, the microcapsule environment allows for self-organization of the cells. Seeding density has also been reported as critical in determining the speed at which spheroids of encapsulated cells arise for other cell types (Bohari et al., 2011). The CHO DP-12 cells in this study did appear to display an aggregated morphology. However closer observation of the colonies (400 and 600 X magnification), suggested that the cells grew in close proximity to each other, maintaining their single cell status, as opposed to adhering together. Animal cells do tend to aggregate. However, the aggregation of cells within microcapsules has been

associated with the formation of necrosis cores due to poor diffusion of nutrients and oxygen to the central cells of the aggregate.

Based on the results of daily cell counts, the growth profiles of cells encapsulated in APA microcapsules, and that of cells cultured freely in suspension, portrayed a similar pattern. A lag period of 1 day was observed prior to exponential growth. Encapsulated cells spent 2 extra days in exponential growth resulting in a maximal viable cell yield of 3.7 fold higher than that which was achieved by the freely suspended cells. The increased yield brought about through encapsulation is somewhat more dramatic than those achieved by Breguet et al., (2007). They reported a maximum viable cells density of $2.2 * 10^6$ cells/ml_{reactor} for encapsulated cultures in comparison with $1.41 * 10^6$ cells/ml_{reactor} for suspension cultures for a CHO cell line. The magnitude of increased cell yield brought about by encapsulation is cell type and line dependent. Awrey et al., (1996) reported that for a series of genetically modified mammalian cells encapsulated in APA microcapsules, the increase in cell number over time varied in the range of 10- to 153- fold, dependent on the cell type. Breguet et al., (2007) did however report on a decreased maximum specific growth rate for the encapsulated cells in comparison to that observed in suspension cultures. There was no significant difference in the maximum specific growth rate displayed by encapsulated and suspension cells in this current study. Similar cellular growth rates for suspension and encapsulated cultures have also been reported for hybridoma cells producing mAbs. Sinacore et al., (1989) noted an increased cell yield in immobilised cultures due to an exponential phase double the length of that observed in suspension cultures. Increased growth periods and maintenance of viability has been associated with the protection microcapsules offer cells from shear forces (Lee and Palsson, 1990). In this current study the stationary phase for both cultures was short, 1 day for suspension cells and 2 days for encapsulated cells. Different cell types display different growth profiles. Hybridoma cells encapsulated in APA microcapsules displayed a 4 day stationary phase which was comparably longer than that displayed when the cells were cultured in suspension (Ma et al., 2006). It may be concluded therefore that cells show an improved growth process in microcapsules. The APA microcapsules in this culture were strong enough to support the increasing cell numbers until the end of the stationary phase. However there was a calculated 18 % loss in microcapsules due to breakage between days 7-9. Although, APA liquid core microcapsules have been widely reported for their support of a range of mammalian cell growth, as noted above, they are however widely associated with instability issues, which could prove detrimental should the culture period wish to be

extended by the introduction of various feeding strategies. Brequet et al., (2007) reported that the liquefaction of solid or gelled core microcapsules results in a decrease in the mechanical resistance properties of the microcapsules. Liquid core microcapsules have been documented as better for the support of cell growth rather than solid core (Zhang et al., 2007), however, a range of factors, including the fact that their core is liquefied by sodium citrate, influence their stability. Alginate viscosity (Koch et al., 2003), concentration and number of PLL layers (Leick et al., 2011), and microcapsule size (Zhang et al., 2007; Brequet et al., 2007) have been some of the parameters put forward which determine microcapsule stability and integrity in cultures. There have been variations in the reported integrity time when used as matrices for mammalian cell culture. Koch et al., (2003) reported that 0.03 % microcapsules remained intact for 8-12 weeks when fully colonised with BHK cells. For APA liquid core with CHO cells and two different types of PLL membranes, Ma et al., (2012) reported that capsules remained intact for > 25 days.

The microcapsules used in this experiment decreased dramatically in mechanical resistance from days 0 (0.49 +/- 0.09 (g/capsule)) to day 3 (0.07 +/- 0.01 (g/capsule)). A similar trend was noted for 500 µm and 800 µm diameter liquid core APA microcapsules by Brequet et al., (2007) for a perfusion culture conducted in a 1.5 L bioreactor. Their results suggested that shearing in the bioreactor was a parameter that influenced microcapsule stability. However it is also important to note that they reported decreased stability brought about by the perfusion experiment conducted. The constant displacement of equilibrium between medium and microcapsules was destructive. The instability was however not a major issue in this study as the cell number had reached a maximum and viability had begun to decrease, due to the batch nature of the experiment, before major capsule breakup was observed.

Microscopic analysis of the capsules also indicated a lack of complete colonisation of the microcapsules. The degree of colonisation was determined to be around ~5 % on day 6 when the maximum cell number reached. The degree of colonisation of cells in microcapsules has been demonstrated to be somewhat varied in literature depending on cell type, line and microcapsule used. The encapsulation of a murine fibroblast cell line in small (350 µm diameter) APA microcapsules resulted in complete colonisation of the capsule after 4 weeks in culture (Bohari et al., 2011). Hybridoma cells encapsulated in both solid and liquid core APA microcapsules completely colonised the capsules within 11 days in culture (Ma et al., 2006). However for a CHO cell line producing endostatin in APA microcapsules, only 50 % colonisation was reported (Zhang et al., 2007). Similarly another CHO cell line was only

capable of colonising 20 % of the capsules volume when cultured in liquefied APA capsules (Brequet et al., 2007). Brequet et al., (2007) reported that capsule size is an important parameter in regards to the promotion of high cell densities inside the capsule matrix. Large capsules (> 1 mM) are often associated with diffusion limitations of critical nutrients resulting in reduced cell growth and necrosis within the capsule core (Glacken et al., 1983; Robitaille et al., 1999). The effect of capsule size on colonisation of the capsules was investigated by Brequet et al., (2007). It was found that in the range of capsule sizes investigated (500-800 μm), colonisation was not significantly affected by size. It was presumed that low values of colonisation were in fact due to an inability of cells to grow freely in the capsule inner core. An incubation of 30 minutes in PLL may be sufficient time for the PLL to penetrate the capsule membrane resulting in polymerisation of the capsule core. Depending on the degree of polymerisation, the growth of cells inside the capsules may have been limited by free available space. Also polymerisation of the capsule core is associated with shrinkage in the size of the capsules due to crosslinking of the alginate (Peirone et al., (1998). The size of the capsules in this current study were, however, in the outer limits of the expected size range for the encapsulation process (700-900 μm) (Brequet et al., 2007). It is therefore possible that this is not a plausible explanation for the lack of complete colonisation observed. Brequet et al., (2007) also investigated the possibility of inhibition of cell growth by alginate. The presence of alginate in the core of microcapsules is thought to inhibit cell growth through electrostatic interactions between the cells and the highly anionic polyelectrolyte. However studies in which cells cultured in the presence of alginate in suspension portrayed an increased doubling time, thus suggestion cell growth and capsule colonisation is not inhibited by alginate. Alginate itself is reported non-cytotoxic (Robitaille et al., 1999). It may therefore be proposed that the only inhibition alginate may induce on cell growth is as a physical hindrance occupying space within the capsule.

It may also important to consider diffusion limitations as a factor restricting complete colonisation of the capsules. However the fact that both carbon sources, glutamine and glucose, were exhausted by the end of the exponential and stationary growth phases, respectively, indicate that further cell growth and colonisation may have been restricted by the depletion of nutrients, glutamine (in particular).

In relation to metabolism encapsulated cells displayed different metabolic profiles in comparison to suspension cultures. As noted above, the growth limiting substrate for the encapsulated cultures was determined to be glutamine, verified by its complete exhaustion at

the end of the exponential growth period. Cells were maintained in the stationary growth phase through the utilisation of glucose.

An evaluation of the consumption profiles of glucose for both suspension and encapsulated cultures suggests that the different modes of cultivation impacted on the utilisation of this carbon energy source by the cells. Both the rate of glucose consumption and the cell specific glucose consumption rate were 60 % and 80 % higher, respectively, in the suspension cultures in comparison to the encapsulated cultures. As a result of increased glucose consumption a 3-fold higher concentration of lactate was accumulated in the suspension cultures. A 50 % higher yield of lactate on glucose occurred over the culture period in the suspension cultures in comparison to the encapsulated cultures. The lower yield of lactate observed in encapsulated cultures has been observed in other CHO cell lines cultivated in APA microcapsules as oppose to in suspension (Brequet et al., 2007). Lower lactate yields indicate more efficient oxidative utilisation of glucose by the cells in microcapsules. Similar results were also noted for other immobilised cell types, including hybridoma cell lines (Ma et al., 2006). The immobilisation of cells facilitated signal transduction and mass transfer in the organised community (Wen-tao et al., 2005).

The second major carbon source in mammalian cell culture, glutamine, was depleted at the end of the exponential phase in encapsulated cultures. It was not completely depleted until the end of the stationary phase in the suspension cultures. However there was a 73 % decrease in the cell specific consumption rate of glutamine in encapsulated cultures in comparison to suspension cultures, suggesting that glutamine was utilised more efficiently by cells cultures in liquid capsules. The decreased cell specific glutamine consumption rate noted in encapsulated cultures is coupled with a decreased cell specific ammonia production rate in the encapsulated cultures. The utilisation of amino acids by encapsulated cells has been noted to be different to that of suspension cells. CHO cells cultured in APA microcapsules have been noted to consume a variety of amino acids, including glutamine at a faster rate than suspension cells. It is proposed that encapsulated cells may grow under a special oxidation stress microenvironment and so cells may undergo great biochemistry changes during the process of microencapsulation and adaptation to 3-D culture conditions (Lv., et al., 2008).

The results may suggest an alternative growth limiting substrate between the two modes of cultivation (glucose for suspension cultures, meanwhile glutamine for encapsulated cultures).

However it must also be considered that the high concentration of lactate may have also been responsible for the rapid decline in viable cell density noted in the suspension cultures.

In regards to protein production by encapsulated cells, no increase in the specific rIgG1 production rate was noted between cells cultured in APA microcapsules and cells grown freely in suspension. The effects of encapsulation on the specific protein production rate displayed by the cells appears to be dependent upon the cell type and line. It has been reported that the immobilisation of hybridoma cells in alginate gel beads does not interfere with the expression of mAbs. Increased protein yields were achieved due to higher cell number producing the protein (Sinacore et al., 1989). However, 2-3 fold increases in the specific recombinant protein production rates have been noted for a CHO cell line when encapsulated in APA capsules in comparison to growth in suspension (Brequet et al., 2007). Increased mAb production was also noted for hybridoma cells immobilised in alginate beads in comparison to when the cells were cultured in suspension (Lee and Palsson, 1990). However, the co-encapsulation of two HEK cell lines producing Vascular endothelial growth factor165b (VEGF_{165b}) and interferon (IFN- α) resulted in a similar production profile to that observed by non-encapsulated cells (Afkhami et al., 2012). The same was observed for the encapsulation of HEK producing VEGF_{165b} on its own (Afkhami et al., 2010). Enhanced volumetric (30-60 fold) and specific productivities (4-8 times) were on the other hand achieved when BHK cells were immobilised in collagen gel fibres (Yamaguchi et al., 1997). Enhancement of the specific production rate through the immobilisation of cells is thought to be due to a number of potential factors. Various commercially available alginates suffer impurities within their structure leading to immobilisation stress. It is proposed that this stress increases the specific productivities displayed by the cells within the bead (Selimoglu and Elibol, 2010). It has been observed in immobilised hybridoma cultures producing mAb, a loss in viable cell density may occur in the first week post-immobilisation due to the stress of the procedure on the cells. However in this time period mAb production continued to increase, thought to be induced by immobilisation stress (Selimoglu et al., 2012). Low growth rates displayed by certain cell lines in immobilised culture conditions has also been associated with increased specific production rates (Yamaguchi et al., 1997). Related to a reduction in growth rate is the concept of reduced genetic drift and production instabilities associated with fast growing cells (Lee and Palsson, 1990). There is also reason to believe that increased specific mAb production is achieved due to localisation of the cells in the immobilised matrix, thus exposing them to higher concentrations of autocrine factors. Hybridoma cells produce

autocrine growth factors which either promote, (IL-6), or inhibit, transforming growth factor β 1 (TGF β 1), mAb production (Lee et al., 1993). As specific growth rates or specific rIgG1 production rates displayed by cells cultured in APA microcapsules were not significantly different to those displayed by cells cultured in suspension, it may be presumed that the encapsulated cells were not influenced by immobilisation stress or the influence of autocrine factors concentrated in the local area.

Productivity was however improved in that an increased volumetric productivity was achieved due to high cell numbers producing the protein. Therefore microencapsulation did prove beneficial for enhanced cell yield and productivity, in comparison to suspension cultures. It should also be noted that the protein was produced into the extracapsular media. The rIgG1 protein has a molecular weight of 150 kDa. The molecular weight cut-off point of the microcapsules is in theory determined by the PLL used, in this case 30,000-75,000 Da. Various studies have reported on the secretion of proteins from immobilisation matrices whose molecular weights are higher than the cut-off point presumed of the matrix. Alginate beads of concentration 0.8 % w/v, containing entrapped hybridoma cells permitted the free diffusion of IgG1 (Sinacore et al., 1989). APA capsules containing madin darby canine kidney (MDCK) cells permitted the diffusion of proteins within a range of 22-300 kDa (Peirone et al., 1998). Similarly, APA microcapsules with a presumed molecular weight cut-off point of <100 kDa, permitted the secretion of protein products with molecular weights in the range of 21-300 kDa (Awrey et al., 1996). As mentioned in the introduction, Section 5.1, the authors suggested that the permeability of the proteins through the membrane of the APA capsule was determined by not just the size of the protein but also its ionic charge, shape and radius of gyration. The same authors also proposed that the encapsulation process itself does also influence the porosity of the microcapsule, regardless of the molecular weight cut-off point of the PLL used. When alginate capsules are first extruded with the cells as a suspension, some cells may become lodged in the periphery of the gelled spheres. The presence of cells on the periphery interrupts the subsequent laminating steps with PLL, thus creating pores in the membrane. This concept was verified by a separate study conducted by the authors in which cells were not mixed with membrane forming solutions. The absence of cells on the periphery maintained the molecular weight of the capsules at the presumed level of >100 kDa.

Confocal analysis of the APA capsules containing cells on day 1 of this current study verifies that cells were present on the periphery of the capsule membrane, as noted in Figure 5.3.1 below.

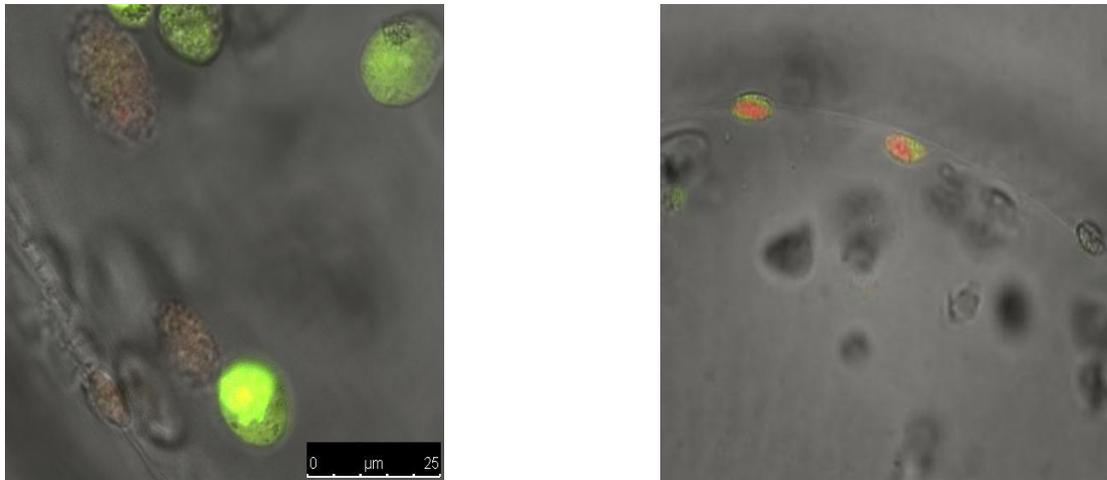


Figure 5.3.1: Confocal analysis of the surface APA microcapsules containing cells sampled on day 1 from culture. Live cells (stained green) appear on the periphery of the capsule, protruding the membrane.

At the beginning of the study, it was hypothesised that the use of encapsulation as a mode of cultivation may also have implications on the quality of the recombinant protein produced. The quality of the recombinant protein is defined in terms of its glycosylation and aggregation state. As noted in the results section, over 95 % of the rIgG1 harvested from culture media was in a monomeric form. Similarly IgG1 harvested from suspension cultures demonstrated to be 99 % in a monomeric form. The results suggest that aggregation of the protein did not occur due to encapsulation. As the protein was secreted, concentration induced aggregation would not have occurred brought about by the presence of high protein concentrations localised within the capsules.

It was also hypothesised that the cultivation of cells in APA microcapsules would affect the growth and productivity of the cells differently to that observed when the cells are cultured in suspension. Increased yields in maximum viable cell density reached and maximum concentration of recombinant protein produced were observed when cells when cells were encapsulated. However the specific rates of growth and productivity were not individually affected. The specific growth rate and production rate of the cells are known to affect the glycosylation of produced recombinant proteins from both a macro- and micro-heterogeneity point of view. This concept has been extensively reviewed in literature (Butler, 2006, Hossler

et al., 2009). Both rates determine the rate of translation of the recombinant proteins, and subsequently the time in which the protein spends in the processing vehicles, such as the endoplasmic reticulum (ER) and golgi apparatus (GA). In the ER, the enzyme oligosaccharide transferase is involved in the attachment of glycans to emerging polypeptide backbones of newly synthesised proteins. Alterations in the activity of the enzyme, often brought about by the length of time the protein spends in the ER gives rise to microheterogeneity. Newly synthesised proteins are transported to the GA where a series of transferase enzymes are involved in the process of attaching residues such as galactose and sialic acids to the glycan structure. Specific rates of growth and productivity are known to affect glycan processing as extensively reviewed by Butler, (2006). Analysis of the heterogeneity of glycan structures harvested from rIgG1 from both encapsulated and suspension cultures indicate that no significant difference in the glycosylation profile of the protein induced by either culture condition. Protein harvested from encapsulated cultures had a slightly reduced (25 %) relative percentage of M5 structures attached at the end of the exponential growth period, indicating more complex glycan processing. However ~2.8-fold increases in the relative % of this glycan did occur over the stationary growth period in the encapsulated cultures. The overall result was that there was no significant difference in the glycoform profile of protein harvested from suspension and encapsulated cultures at the end of the stationary growth period. Such results may be expected based on the similar specific growth and production rates displayed by the cells when cultured under both conditions. Increased levels of M5 are associated with reduced levels of complex glycan processing. Complex glycan processing relies on the availability of uridine di-phosphate N-acetylhexosamine (UDP-GNAc) for the transfer of N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) residues to the non-complex glycan structure in the Golgi apparatus. A decrease in UDP-GNAc is associated with limiting levels of glucose and/or glutamine. An evaluation of the incorporation of glucose and glutamine into the glutamine:fructose-6-phosphate (GFAT) network, as described by Nyberg et al., (1999) describes such an effect of glucose and glutamine limitation. Due to the batch nature of both cultures in this experiment, both glucose and glutamine did become limiting. The metabolism of glucose and glutamine in the encapsulated and suspension cultures was noted to be different in that glutamine was depleted at the end of the exponential phase in encapsulated cultures and glucose by the end of the stationary phase. The opposite was noted for suspension. The increase in M5 noted at the end of encapsulated culture in comparison to that

observed at the end of the stationary phase may be attributed to the decrease available for glutamine in the GFAT network over the stationary growth phase resulting in overall lower Glucosamine 6-Phosphate for UDP-GNAc synthesis. The synthesis involves the conversion of Glucosamine 6-Phosphate to N-acetylglucosamine 1-P. The N-acetylglucosamine 1-P is converted to UDP-GNAc using uridine triphosphate (UTP). Glucose is necessary for UTP synthesis. UTP synthesis relies on the entry of pyruvate (from glucose) into the TCA cycle resulting in aspartate production, which is necessary for UTP synthesis as illustrated by DeFrancesco et al., (1976). As glucose was not limiting in the encapsulated culture until the end of stationary phase, then there would have been more UTP. However there would have already been a depletion of N-acetylglucosamine 1-P due to the glutamine depletion. This is a suitable explanation for the increase in the relative % of M5 throughout the stationary phase in the encapsulated culture. The relative % of M5 is quite low in comparison to all other glycans in general. On a positive note, the increase in M5 over the encapsulated culture did not significantly reduce the relative % of the more complex glycans which are usually present at high levels.

In the suspension culture, glucose was limiting over the stationary phase. It must be noted that this stationary phase was however relatively short and that the glucose limitation may not have affected the intracellular UTP for the short amount of time to result in an increase in M5 that was noted for encapsulated cultures which had a longer stationary phase to experience the glutamine shortage resulting in an impact on product quality. These results have shown how the length of time that the shortage of a metabolite occurs affects the quality of the protein.

5.4: Conclusion

The encapsulation of a specific CHO DP-12 cell line producing rIgG1 in liquefied APA microcapsules, 300 μm in radius did not enhance the specific growth rate or rIgG1 production rate in comparison to that which is observed when cells are cultured in suspension. The results are therefore consistent with those that propose that the effect of immobilisation as a technique for enhanced cellular growth and production may be cell line specific. The growth pattern displayed by the cells was however to some extent affected in that the length of time encapsulated cells spent in exponential and stationary growth phases was increased in comparison to suspension cultures. Encapsulation did therefore provide a mechanism for the achievement of higher cell yields ($\text{cells/ml}_{\text{reactor}}$) in comparison to those

achieved when cells are cultured in suspension. Due to the increased number of cells producing the recombinant protein, an increase in the volumetric productivity and final yield of rIgG1 was achieved in encapsulated cultures. There was no significant difference in the quality of the recombinant protein produced at the end of the stationary growth period when cells were cultured in APA microcapsules in comparison to when cells were cultured in suspension. Such results signify that increased cell and product yields may be achieved through encapsulation without a trade-off in the quality of the recombinant protein produced. The principle that alterations in the growth or productivity of immobilised cells may alter product quality is something which other researchers have failed to address. This study did however interestingly show that the relative % of the non-complex high mannose M5 glycan does increase more dramatically throughout the stationary growth period in encapsulated cultures in comparison to suspension cultures. This could therefore become a potential issue should culture conditions be manipulated which result in an increased stationary phase. This study has identified the necessity for maintenance of key metabolites glucose and glutamine, which act as nucleotide sugar precursors for glycosylation, during all culture periods.

A lack of complete colonisation of the capsules (~5 %) was observed for the CHO DP-12 cell line cultured in the liquefied APA microcapsules. This was attributed to the depletion of the carbon energy sources L-glutamine and glucose, as opposed to inhibition of cell growth due to capsule size, presence of alginate or diffusion limitations. The operation of the encapsulated cultures in a perfusion mode of operation would serve to alleviate the growth limitation due to substrate depletion. Perfusion would also prove beneficial in the removal of metabolic waste products. Although the production of such waste products did not prove inhibitory for encapsulated cultures, the enhanced culture longevity envisaged to be brought about through perfusion would result in increased by-product accumulation if media was not removed. The microencapsulation system provides an excellent and economical means of cell retention, facilitating ease of operation for perfusion cultures.

Chapter 6: Application of a control-fed perfusion strategy for enhance colonisation of Alginate-poly-L-lysine-alginate with CHO DP-12 cells; Implications on rIgG1 quality

Abstract

In order to enhance the colonisation of APA microcapsules with a recombinant CHO DP-12 cell line, further than that observed in previous batch cultures (5 %), a control fed-perfusion strategy was designed. The strategy successfully enabled the concentration of the nutrients glucose and glutamine to be maintained within the range required by the growing cells, while the removal of spent medium prevented the accumulation of high concentrations of metabolic waste products, lactate and ammonia. The culminating result was an increase in the degree of capsule colonisation, which was determined to be in the range of 27 % of the total microcapsule volume. Maximum cell numbers per ml_{culturevessel} were noted to increase ~ 10 fold in comparison to batch suspension cultures. Volumetric productivities were increased 2.6 –fold in comparison to other batch encapsulated cultures. During extended growth periods observed in control-fed perfusion cultures in comparison to batch cultures, protein quality improved, denoted by an increase in the relative % of complex glycans present on rIgG1 harvested. There was 73 % and 36 % increases in the relative % of double galactosylated glycans, F(6)A2G2 and A2G2 respectively, for the control-fed perfusion culture in comparison to the batch culture. A limitation in the success of the study was however noted by a decrease in the level of complex glycans over the course of the stationary phase in the control-fed perfusion cultures. This was attributed to the higher total cell numbers present in this culture. The cell viability was not dramatically low (~63 %) at any point during the stationary growth phase. However the use microcapsules as effective cell retention systems prohibited the removal of dead cells from the culture environment, highlighting a limitation in the use of microcapsules in perfusion systems. Dead cells release glycosidase enzymes which cleave complex glycan structures reducing the overall quality of recombinant protein produced. The study has however also highlighted the advantages of encapsulation as a mode of cultivation in combination with a perfusion strategy to enhance cell and product titres and protein quality up to a specific time point.

Abbreviations:

APA	Alginate-poly-L-lysine-alginate
CHO	Chinese hamster ovary
CMP-NANA	Cytidine-5'-monophospho-Nacetylneuraminic acid

CO ₂	Carbon dioxide
EPO	erythropoietin
ER	Endoplasmic reticulum
GA	Golgi apparatus
GalNAc	N-acetylgalactosamine
GFAT	glutamine;fructose-6-P amidotransferase
GlcNAc	N-acetylglucosamine
GU	Glucose unit
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
IFN- γ	Interferon-gamma
IgG1	Immunoglobulin-1
Non-CO ₂	non-Carbon dioxide
OUR	Oxygen uptake rate
PAT	Process analytical technology
SEC	Size exclusion chromatography
UDP-GNAc	Uridine Di Phosphate-N-acetylhexosamines
UDP-GalNAc	Uridine Di Phosphate-N-acetylgalactosamine
UDP-GlcNAc	Uridine Di Phosphate-N-acetylglucosamine
UTP	Uridine Tri Phosphate

6.1: Introduction

A primary objective for the production of monoclonal antibodies on an industrial scale is sustained productivity at high cell densities. Various approaches have been investigated involving the alteration of environmental culture conditions for enhanced growth and productivity of suspension cells in culture (Fox et al., 2004; deZengoitia et al., 1998). Aside from the manipulation of culture conditions, cell immobilisation has been noted as an effective strategy for the enhancement of maximum viable cell densities and increased specific and/or volumetric productivities for a range of cell lines (Sinacore et al., 1989; Lee and Palsson 1990; Awrey et al., 1996; Brequet et al., 2007). For the particular CHO DP-12 cell line producing recombinant IgG1, encapsulation of the cells in APA microcapsules and cultivation under batch conditions resulted in 3.7- fold increases in the maximum viable cell number attainable and volumetric production, in comparison to cells cultivated in suspension, as documented in Chapter 5 of the thesis. However due to the batch nature of this experiment,

maximum cell density was achieved at ~ 5 % degree of colonisation of the microcapsules. An obvious step forward from this study would be to identify a suitable feeding strategy, which would alleviate the nutrient limitations leading to a cessation of the microcapsule colonisation. The four major cultivations methods for mammalian cell culture are: batch, fed-batch, continuous and perfusion (Xie and Wang, 2006), and have been described in the Introduction Chapter, Section 1.2.6. Presented below is an overview of the main advantages and disadvantages to each of the methods in order to devise a suitable feeding strategy to apply to the encapsulated cell cultures.

Batch cultivation of mammalian cells is considered one of the simplest modes of cultivation and is particularly effective when protein expression is growth associated (Gugerli, 2003) and as reviewed in the Introduction Chapter, Section 1.2.6. However, one trait of mammalian cells in culture which is not satisfied by a batch mode of cultivation is their highly deregulated metabolism. Under batch cultivation conditions this characteristically results in the rapid exhaustion nutrients, accumulation of toxic metabolic by-products (namely lactate and ammonia), resulting in the consequent initiation of programmed cell death or apoptosis very shortly after the attainment of maximum cell number. The level of glycosylation has been adversely affected by glucose starvation, the point at which glucose becomes limiting (Chapman and Calhoun 1988; Hayter et al., 1992). Both glucose and glutamine depletion have an impact on the microheterogeneity of the attached glycans (Nyberg et al., 1999; Wong et al., 2004). Waste product accumulation, associated with batch cultivation also affects the microheterogeneity of attached glycans. The effect increased ammonia concentrations in cultures have on the degree of sialylation has been particularly well documented. (Butler, 2006; Hossler et al., 2009; Jenkins et al., 1996). The sharp fall off in viability associated with batch cultivation has been associated with adverse release of glycosidase enzymes into the culture environment, resulting in yields of protein with characteristically compromised integrity and quality (Yang and Butler, 2000; Gramer and Goochee, 1993). Solutions to the aforementioned problems have focused on the development of nutrient feeding schemes in an effort to minimise nutrient fluctuations and reduce waste accumulations, namely fed-batch and continuous (perfusion) mode of cultivation (Chon and Zarbis-Papastoitsis, 2011; Bonham-Carter and Shevitz, 2011; Gugerli, 2003; Gambhir et al., 2003).

The fed-batch strategy seeks to avoid early complete depletion of key media components, common to batch cultivation, by feeding highly concentrated nutrient feeds to the culture at set points. Two methods for the optimisation of the concentration of nutrients in the feed

medium have been proposed and reviewed in the Introduction Chapter, Section 1.2.6. The major advantages and disadvantages of this feeding strategy have also been outlined in Introduction Chapter, Section 1.2.6. Although fed-batch processes have been associated with higher final volumetric product concentrations, in comparison to continuous or perfusion modes (Hu et al., 2011), this principle may have detrimental effects on the product quality. Increased culture longevity gives cause for concern regarding the length of time the protein remains in the culture environment and may be subjected to concentration dependent aggregation. Also, if harvest time is not optimised and cell viability becomes compromised, the risk of the protein being exposed to proteases and glycosidase enzymes is greatly increased. The result may lead to high yields of poor quality product (Gramer and Goochee, 1993). In a fed-batch process toxic waste products are expected to accumulate to higher concentration than observed in a batch culture due to the addition of metabolites beyond the concentration present in the initial medium. Various studies have been conducted in which the presence of high concentrations of metabolic by-products have been noted to be detrimental to glycosylation of recombinant proteins (Hossler et al., 2009; Yang and Butler, 2000; Jenkins et al., 1996).

The problems associated with fed-batch modes of cultivation may be alleviated with the progression to a continuous or perfusion feeding strategy. The main advantages of continuous/perfusion modes of cultivation are that it can avoid nutrient limitation, while reducing the concentration of growth inhibitory metabolites (Yang et al., 2000 and Konstantinov et al., 1996). Product quality and purity may be enhanced, not only due to the reduced byproduct concentration, but also as an effect of the continuous withdrawal of protein containing spent media from the culture environment, thus reducing the overall residence time of the protein in the culture environment in which it may be exposed to proteases and glycosidase enzymes (Rastilho et al., 2002; Ryll et al., 2000). The method of operating a continuous culture and the relevant equations governing the achievement of steady state of cells in such environments have been explained in depth in the Introduction Chapter, Section 1.2.6. The main shortcoming of a continuous mode of cultivation is the relatively low cell concentrations attainable at steady-state due to the necessary bleed rate (Vits and Hu, 1992). A continuous process can only compete economically with batch cultures if a higher cell density and high protein concentrations can be reached. Cell concentration and volumetric productivity can be effectively increased by cell recycle or partial or complete cell retention. Coupling a cell retention device to a continuous bioreactor,

in what is frequently called a perfusion bioreactor, can lead to a 5-10 fold increase in cell concentration (Vits and Hu 1992).

A diverse set of devices have been developed over the years which enable the retention of cells in bioreactors, while minimising damage to the cells. Some of such devices work better than other and have been extensively reviewed by Gugerli, (2003), Chon and Zarbis-Papastoitsis, (2011), Bonham-Carter and Shevitx, (2011) and Voisard et al., (2003). For cells cultivated in suspension, separation is usually based on either filtration, gravity settling or centrifugation (Bonham-Carter and Shevitx, 2011). The performance of cell separating devices relies on a set of five physical or chemical characteristics of cells as particles including: 1) size, 2) density, 3: electrical charges, 4) dielectric constant 5), surface properties (Voisard et al., 2003). A range of filtration devices are available commercially for the retention of cells in bioreactors based on their size. These include: cross-flow filters, hollow fiber filters, controlled shear filtration, vortex flow filters and spin filters. All have been extensively reviewed (Voisard et al., 2003). Benefits associated with such filtration devices include the selective removal and concentration of a particular product (Langes, 2011). Although filtration devices, such as spin filters in particular, were one of the first devices used in perfusion, they have been largely phased out due to the limited scale up potential and reliability. Bioreactor volumes scale up by the cube of the radius. An internal attached filter would therefore require scaling up by the square of it's radius, resulting in a large portion of space inside the vessel being taken up by the filter (Bonham-Carter and Shevitx, 2011). Although external filters may overcome the issue with regards to occupation of internal reactor space, they are considered costly and provide no mechanism for the return or recycle of cells from the spent medium back into the reactor in order to maintain a high cell density (Hu and Aunins 1997). As well as a lack of scale up potential, the problem of membrane clogging or fouling has been widely reported (Blasey and Jager, 1991; Buntmeyer et al., 1994).

The retention of cells inside a bioreactor during a perfusion culture may also be achieved based on the density of the cells. Devices enabling the sedimentation of cells include gravity settlers, centrifuges, hydroclones and centrifugal bioreactors. Gravitational settlers, often present in external loops, are one of the simplest cell retention devices suitable for cells that are shear sensitive. Such devices are more effective for viable cells than dead cells, enabling the delivery of a viable cell free harvest stream (Voisard et al., 2003). However the main drawback of the method is the significant amount of time cells spend in the suboptimal

external loop within the settler (Bonham-Carter and Shevitz, 2011). Enhanced sedimentation may be brought about by centrifugation. However the implementation of such devices may compromise the main beneficial properties of sedimentation as a retention device due to the introduction of shear brought about by the moving mechanical parts (Ryll et al., 2000).

Immobilisation or entrapment of cells within a matrix, such as alginate-poly-L-lysine (APA) microcapsules is a more favorable proposed as a mechanism for the retention of cells in perfusion bioreactor. We have seen in Chapter 5 how encapsulation has been beneficial in offering an efficient protection system for the growth of cells within a 3-D matrix. However a second beneficial property of encapsulation is in the provision of an effective mechanism for retaining cells within a bioreactor without the requirement of a separation device (Brequet et al., 2007). Cells may be retained inside the bioreactor while being protected from hydrodynamic shear created by agitation (Seifert and Philips, 1997). It is proposed that the benefit of the ease of cell retention in the bioreactor, coupled with the protective action of the microcapsules against shear forces would enable perfusion to provide recombinant proteins with fewer variations in glycosylation, thus more consistent in biological activity (Langer, 2011). Cell retention by immobilisation using both macroporous microcarriers and encapsulation has been previously investigated (Goldman et al., 1998; Wang et al., 2002; Brequet et al., 2007; Seifert and Philips, 1997).

It has been explained in the Introduction Chapter, Section 1.2.6 that if cells are completely retained, no steady state should be reached and the cell concentration should go towards infinity. In practice, however, Gugerli, (2003) described how cells grow until one or several nutrients become limiting. When cells are typically cultivated in situations of complete retention the characteristic behaviours of cells may be summarised as 4 distinct phases: (1) a first exponential growth phase due to excess nutrients (2) a second linear growth phase limited by the feed rate, (3) a decrease in growth due to insufficient nutrient feeding (4) slow cell death due to apoptosis (Gugerli, 2003).

In the past two decades, efforts have been made to optimise the criteria governing a perfusion process, which include 1) the time point at which perfusion is initiated, 2) the magnitude of the perfusion rate, 3) the extent to which the cells are retained (Vits and Hu, 1992; Dowd et al., 2001; Wang et al., 2002; Konstantinov, 1996; Golmes, 1998; Meuwly, 2004), as noted in the Introduction Chapter, Section 1.2.6 However even with the optimisation of such criteria, there are 2 costly disadvantages associated with altering the perfusion rate. Increasing the perfusion rate results in the consumption of large volumes of medium, which increases the

overall cost and labour requirements of the process. The resulting harvest stream emerging from the culture contains very dilute concentrations of the recombinant protein (Gugerli 2003). Although this is advantageous in comparison to fed-batch cultivation modes, in which high levels of the protein remain in the reactor for extended periods of time, the result of the perfusion operation usually requires increased complex purification processes. The product concentration in the emerging harvest stream is often lower than that which is present in a typical fed-batch culture (Ozturk, 1996). This can often translate into higher failure rates than a typical fed-batch process (Chon and Zarbis-Papastoitsis).

A different mode of cultivation, namely controlled-fed perfusion, seeks to address these issues and encompasses the advantages of both perfusion and fed-batch. To alleviate the product dilution in perfusion cultures, noted above, perfusing cultures at lower rates using a medium fortified with specific nutrients (such as glucose or glutamine) has been demonstrated to be successful (Ozturk 1994; Runstadler et al., 1992). Banik and Heath, (1996) also suggested that a more appropriate perfusion mechanism would involve one that would increase the level of specific nutrients in the feed medium as opposed to increasing the rate at which the medium is perfused. However an underlying issue in this mechanism was noted by the high concentration of by-products which accumulated in the culture. Yang et al., (2000) sought to minimise this byproduct accumulation, while adhering to the controlled-fed perfusion mode of cultivation. Their strategy involved making a forward rate adjustment to the perfusion stream, in accordance with the predicted cell number and their nutrient demands for the next day. The perfusion stream was fortified with glucose at a concentration beyond that which was usually present in the medium formulation. The result was a 9.5- fold and 1.8- fold increase in viable cell number in comparison to that achieved for batch and perfusion cultures respectively. The yield of recombinant protein in such controlled-fed perfusion cultures was in the range of 10- fold and 2.5- fold higher than batch and perfusion cultures respectively. Variations of this method have been recently published in which Teng et al., (2011) conducted a controlled fed-perfusion culture in which the perfusion stream was accompanied by a separate feed stream containing key nutrients in concentrations required by the cells, as determined by the consumption rates displayed by the cells. Cell growth exceeded 3.8- fold higher than that observed in batch cultures and over a 2- fold increase in the concentration of recombinant protein product.

In this current study a variation of the controlled fed-perfusion strategy was designed in order to assess if further cell growth resulting in increased colonisation, beyond that observed in the

batch encapsulated cultures in Chapter 5, could be achieved. The strategy involved maintaining the perfusion rate to a set limit of 1 or 2 medium changes per day, in which the volume of media removed was a quarter of the total culture volume and the same volume of media replaced. The number of medium changes per day was defined by both the accumulation of by-product and also the mechanical and labour resources available. Daily sampling and spent media analysis would enable determination of cell number, and glucose, glutamine and ammonia concentrations which would be used to determine the growth rate and cell specific consumption and production rates. The concentration of the relevant metabolites to add to the feed media was adjusted in accordance with a projected cell density for the next day, to meet the predicted nutrient demands. The model was initially proposed based on the data accumulated from the batch encapsulated cultures carried out in Chapter 5. The main aim of the study was to determine if enhanced capsule colonisation could be achieved, beyond that achieved in batch cultures (5 %) in Chapter 5, by eliminating nutrient limitations which were identified in the batch cultures and associated with growth cessation. The underlying aims of the study were to identify whether the applied control-fed perfusion strategy would in turn not only affect colonisation but also all cellular growth, metabolic and production activities for the, Chinese hamster Ovary (CHO), CHO DP-12 cell line producing rIgG1. An overall aim of the study was to determine if any altered cellular activities induced by the control-fed perfusion strategy would in turn impact of the quality (glycosylation and/or aggregation state) of the recombinant Immunoglobulin 1 (rIgG1) protein produced.

6.2. Results:

To evaluate the potential for a perfusion strategy to enhance the colonisation of APA microcapsules, beyond that observed in Chapter 5 (5% colonisation), a number of studies were conducted. In section (6.2.1) initial efforts involved the application of a controlled-fed perfusion strategy to the CHO DP-12 cell line encapsulated in APA microcapsules and used to inoculate a 1.7 L minifors reactor at a ratio of 300 ml capsules: 900 ml media. The controlled-fed perfusion strategy involved offline determination of cell number and metabolite concentrations, namely glucose, glutamine and ammonia on a daily basis. The results of the evaluated parameters were then used to determine the growth rate and specific consumption and production rate displayed by the cells for the named metabolites. Subsequently a forward adjustment was made in the concentration of the metabolites in the perfusion medium in accordance with the projected cell density for the next day, to meet the

predicted nutrient demand. In this particular study the perfusion rate was not adjusted due to the untimely cessation of the culture as a result of capsule instabilities. Perfusing of the reactor involved the removal of 300 ml of spent media and the addition of 300 ml of fresh media (containing glucose and glutamine concentrations required by the actively growing cells). Simply adjusting the concentration of the named nutrients in the perfusion medium was sufficient to meet the cellular demands. (6.2.2) In order to overcome the instability issues of the microcapsules in the 1.7 L minifors reactor, an investigation was conducted in which CHO DP-12 cells encapsulated in APA microcapsules were cultured in the less shearing 1 L Erlenmeyer shake flask environment. Before the transition could be made to such a platform using the perfusion strategy, it was first necessary to identify if of all cellular activities were comparable to that observed when cells were cultured in APA microcapsules in the 1.7 L minifors reactor platform under batch culture conditions. The perfusion strategy was designed based on the analysis of the behaviour of cells cultured in APA microcapsules under batch cultivation conditions in Chapter 5. The results in Chapter 4 did indicate that cellular activities were altered when cells were cultured in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor when in suspension. The cultivation of the cells in APA microcapsules under batch cultivation conditions in the 1L Erlenmeyer shake flask platform was completed to ensure that any alterations noted in cellular activities after application of the perfusion strategy to cells cultured in the 1 L Erlenmeyer shake flask platform, were due to the applied perfusions strategy and not due to the change to a 1 L Erlenmeyer shake flask platform. In section (6.3.3), after all cellular activities of the CHO DP-12 cells encapsulated in APA microcapsules had been characterised when cultured under batch cultivation conditions in the 1 L Erlenmeyer shake flask platform, it was necessary to apply the controlled-fed perfusion strategy to this platform. To date, cells cultured in 1 L Erlenmeyer shake flasks have been done so in unvented shake flasks in a non-Carbon dioxide (non-CO₂) incubator. Due to the possibility of mass transfer issues in such an environment, the perfusion strategy was applied to cells cultured in both the unvented shake flasks in a non-CO₂ incubator and also cells cultured in vented shake flasks in a 5 % Carbon dioxide (CO₂) incubator. The ratio of capsules to media in all 1 L Erlenmeyer shake flask cultures was comparable to that used in the 1.7 L minifors reactor cultures (100 ml capsules: 300 ml of media). Perfusing of the cultures in unvented shake flasks was inline with that completed in the 1.7 L minifors reactor culture, with the perfusion volume adjusted to 100 ml. It was necessary to complete this

procedure twice daily from day 9 onwards in the vented shake flasks due to an increase in ammonia accumulation.

6.2.1: Batch encapsulated –v- Perfusion encapsulated in 1.7 L minifors reactor

In order to investigate the potential for optimised colonisation of APA microcapsules with a CHO DP-12 cell line, a controlled-fed perfusion strategy was designed and applied to cells cultured in a 1.7 L minifors reactor platform. The perfusion strategy was designed based on the results of cells cultured in APA microcapsules under a batch mode of cultivation in the 1.7 L minifors reactor. In such batch cultures it was noted that a cessation in the growth of cells coincided with a depletion of the nutrients (glutamine and glucose). The perfusion culture was operated as a batch culture until day 5, at which point offline cell growth and metabolite analysis suggested that the concentration of the key metabolites glucose and glutamine was insufficient to meet the requirements of the exponentially growing cells. A forward adjustment was made to the concentration of the named metabolites in 300 ml of fresh culture media in order to meet the demands projected cell density for the next day. The 300 ml of fresh media was added to the bioreactor following the removal of 300 ml of rIgG1 containing spent media. The procedure was applied to the culture until cessation on day 9, due to a break down in the capsule structure.

6.2.1.1 Characterisation of microcapsules

6.2.1.1.1 Capsule size:

The APA microcapsules were formed by the extrusion of a sodium alginate solution through a 300 µm nozzle. Due to liquefaction of the alginate core, swelling of such microcapsules in the range of 150-200 % of the original volume has been reported (Breguet et al., 2007). Based on previous studies, the final diameter of the microcapsules would be expected to be in the range of 700-900 µm.

Thirty capsules were measured individually in order to calculate the average diameter and the standard deviation. Generally the size distribution was in the range of 3-5 %.

Figure 6.3.1.1.1 below illustrates the capsules radius size over the cultivation period for both the batch (Chapter 5) and perfusion cultures. The results presented below for the batch cultures are the average of triplicate cultures. Capsule size throughout the perfusion culture is consistent with that observed during the batch cultures. Again, it is important to note, that

size was only determined for intact capsules. The evolution of broken capsules towards the end of the culture period was omitted. It is estimated that the number of capsules present in the reactor during the perfusion study decreased by a total of 58 % from days 7-9. However the loss of capsules due to breakage between days 7-9 in the batch cultures was estimated at ~18 %. A combination of media change during perfusion and also the increased number of cells/ml capsule serve as plausible explanations for the increased capsule instability observed in the perfusion culture.

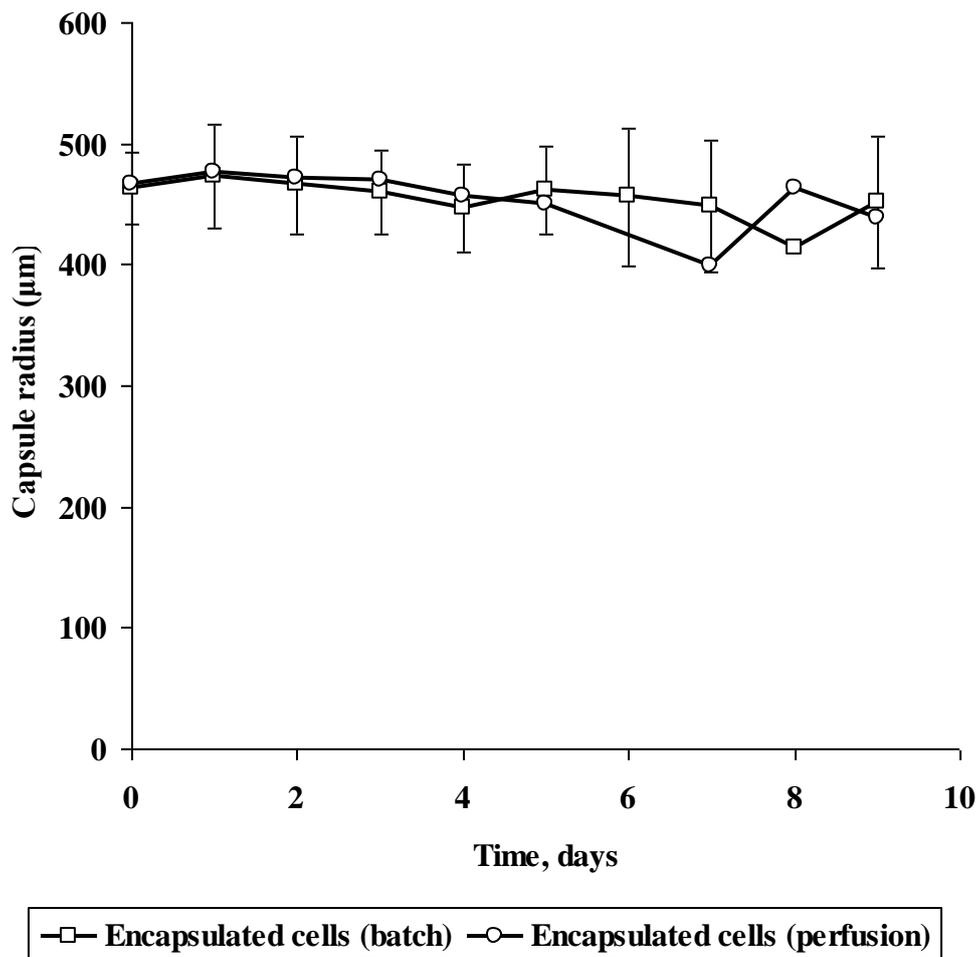


Figure 6.2.1.1.1.1: Average capsule radius size (μm) \pm STD over the duration of the cultivation period for each of the three batch encapsulated cultures completed and the single control-fed perfusion culture completed. The size of approximately 30 capsules was measured at each time point and an average determined.

6.2.1.1.2 Capsule strength

The mechanical strength of the microcapsules in the batch and control-fed perfusion cultures was routinely evaluated in terms of Force (g)/capsule which the capsule could withstand using the Texture analyser.

Evaluation of Figure 6.2.1.1.2.1 below allows for comparisons to be made between the mechanical resistance of microcapsules present in batch and perfusion cultures performed in the 1.7 L minifors reactor culture. Chapter 5 previously stated that for batch cultures, a dramatic decrease in capsules strength may be noted over days 0-3 in the cultures. This may be attributed to the introduction of the capsules to the shearing environment of the bioreactor and also equilibration of the capsules with media. As the control-fed perfusion culture essentially acted as a batch culture from days 0-5, it would be expected that there would be no significant difference in the mechanical resistance of the microcapsules under both conditions during this time period. This may be observed in Figure 6.2.1.1.2.1 below. Once the perfusion strategy is applied, the mechanical resistance of the microcapsules may be potentially unfavourably affected by the necessity of further equilibrations with the fresh media. No further significant decreases in microcapsule resistance were noted from day 5 onwards in comparison to the batch. The loss of microcapsules from day 7 onwards due to breakage in the control-fed perfusion culture may be attributed to the incapability of already weakened microcapsules to withstand the combination of higher cell number ($\text{cells/ml}_{\text{capsule}}$), the equilibrium required with the fresh medium and the shear forces of the bioreactor environment. Instead of seeing further decreases in the mechanical resistance, vast capsule breakage is noted. At such low mechanical resistances, capsule breakage was also reported in the batch cultures, however not to the same degree as that observed in the control-fed perfusion cultures.

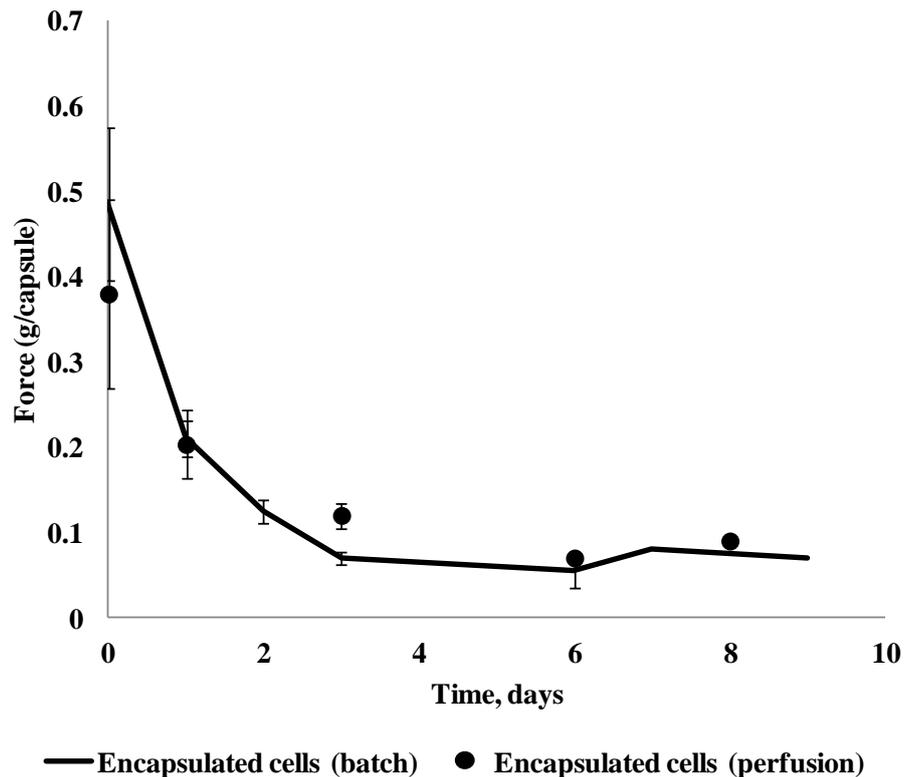


Figure 6.2.1.1.2.1: Mechanical resistance (force (g)/capsule) over cultivation period for 300 μm APA microcapsules containing CHO DP-12 cells cultivated in a 1.7 L minifors reactor under batch and control-fed perfusion conditions. A total of 3 layers of microcapsules were analysed on a daily basis for each of the cultures carried out. Values presented are average of such triplicate analysis \pm STD for one of the batch cultures performed and the one control-fed perfusion culture performed.

6.2.1.2.: Growth of encapsulated cells under batch and perfusion modes of cultivation

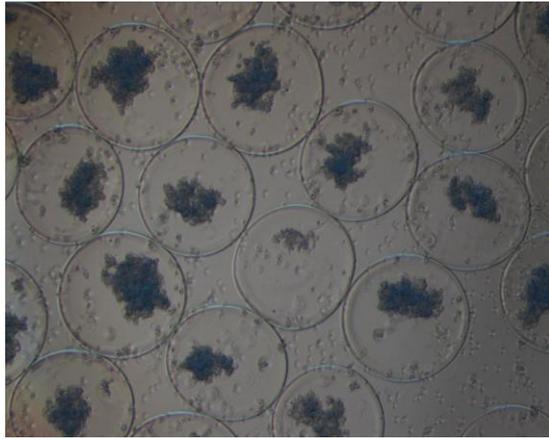
In order to investigate if a designed controlled-fed perfusion strategy has the potential to enhance the colonisation of a CHO DP-12 cell line cultured in APA microcapsules, in comparison to that which is observed under batch cultivation conditions, it was necessary to investigate the growth of cells inside the capsules and cell densities reached in terms of both $\text{cells/ml}_{\text{capsules}}$ and $\text{cells/ml}_{\text{reactor}}$.

6.2.1.2.1 Growth of cells inside microcapsules

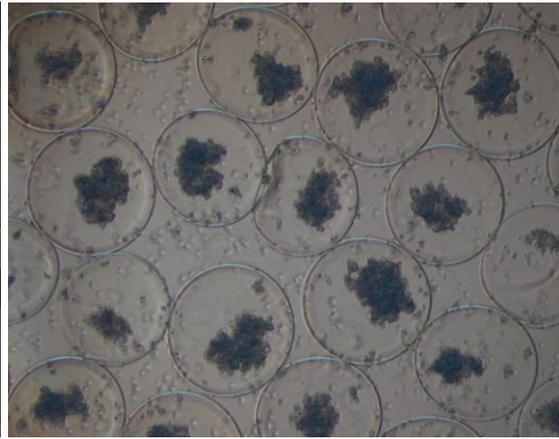
CHO DP-12 cells were used to inoculate 300 ml of alginate at a seeding density of 0.5×10^6 $\text{cells/ml}_{\text{alginate}}$. Such microcapsules were used to inoculate a reactor at a ratio of 300 ml capsules: 900 ml of media. The reactor operated in batch and/or controlled-fed perfusion mode for a period of 9 days, with perfusion initiating on day 5 of the culture period. The

growth pattern displayed by cells cultured in batch conditions was extensively evaluated in Chapter 5 and may be reviewed in Figure 6.2.1.2.1.1 below in which the colonisation of the microcapsules with cells on days 6 (one day after cessation of exponential growth) and 8 (end of stationary phase of growth) are portrayed. The degree of colonisation of the capsules with cells was determined to be 5 % on day 6 of the batch culture. The beneficial effect of a controlled-fed perfusion strategy, designed to eliminate a limitation in two key nutrients (glucose and glutamine) may also be noted by evaluation of Figure 6.2.1.2.1.1. Colonisation of the microcapsules in the controlled-fed perfusion culture may be observed and it may be qualitatively determined that the designed perfusions strategy did enable enhanced colonisation of the microcapsules. The degree of colonisation of the microcapsules on day 8 of the perfusion culture was calculated as 9.3 %. Therefore there was an 86 % increase in the degree of colonisation brought about by the perfusion strategy applied.

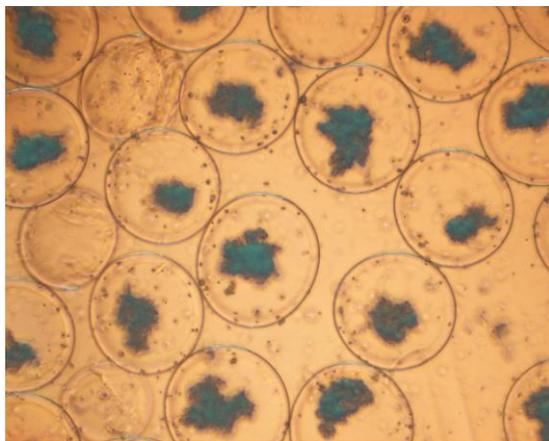
Figure 6.2.1.2.1.2 below allows comparison between the growth of cells in APA microcapsules in the 1.7 L minifors reactor under batch and controlled-fed perfusion strategies in terms of the viable cell density per $\text{ml}_{\text{capsules}}$ over the cultivation period. Cell counts were performed using the trypan blue exclusion method and haemocytometer. Prior to performing cell counts, microcapsules were ruptured by extrusion through a 300 μm needle and diluted in PBS. Under a batch mode of cultivation, evaluated in Chapter 5, colonisation of the capsules continued until day 6. The data portrayed for the batch cultures is based on the average of triplicate cultures \pm the standard deviation. Cessation of exponential growth at the maximum displayed growth rate did cease on day 5, followed by a short stationary phase and subsequent onset of cell death. Cells continued to colonise the capsules until day 8 of the controlled-perfusion culture resulting in the attainment of a maximum viable cell number ($\text{cells}/\text{ml}_{\text{capsules}}$) 36 % higher than that observed in the batch cultures, as noted in Table 6.2.1.2.1.1.



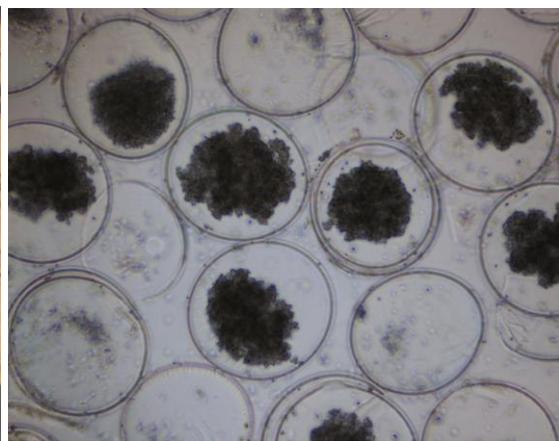
Batch day 6



Perfusion day 6



Batch day 8



Perfusion day 8

Figure 6.2.1.2.1.1: Microscopic analysis (40 X) (unstained) of microcapsule colonisation by cells on cultivation days 6 and 8 in both batch and perfusion cultures. Cells were originally seeded at $0.5 * 10^6$ cells/ml alginate and which was extruded through a 300 μ m nozzle as part of the liquid core microcapsule formation. The resulting microcapsules were used to seed a reactor with a ratio of 300 ml capsules and 900 ml media.

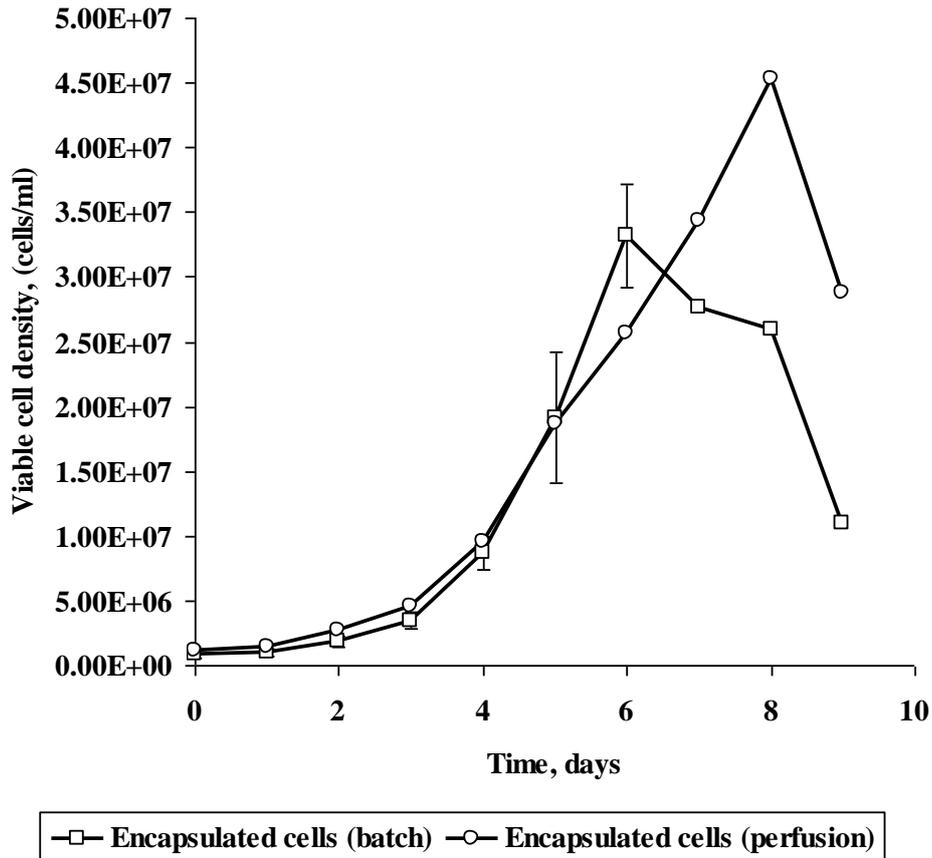


Figure 6.2.1.2.1.2: Growth of CHO cells cultured in 300 μm liquid core APA microcapsules under batch and perfusion modes of cultivation. The cell concentration is given with respect to the capsule's volume. Cultures were seeded at an average viable cell density of 0.3×10^6 cells/ml_{reactor}. Cell numbers were enumerated on a daily basis using trypan blue exclusion method and a Neubauer haemocytometer. Encapsulated cells were liberated prior to counting via extrusion of a capsule/PBS suspension through a 300 μm needle 3 times. Duplicate cell counts were performed. Values presented above are the average of triplicate cultures carried out in parallel \pm STD, for the batch encapsulated cultures performed. Values for the perfusion culture represent those obtained for the single culture carried out.

Table 6.2.1.2.1.1: Calculated maximum viable cell densities with respect to per ml capsule for cells cultured under batch and perfusion modes of cultivation in a 1.7 L minifors reactor culture. Max VCD for the batch cultures is the average of triplicate cultures carried out in parallel, +/- STD. Value for the perfusion culture is that which was achieved for the single culture performed

Culture	Max VCD (*10 ⁶ cells/ml _{capsules})
Encapsulated cells (batch)	33.2 +/- 3.95
Encapsulated cells (perfusion)	45.3

6.2.1.2.2. Viable cell numbers per ml_{reactor}

Microcapsules for both batch and perfusion cultures were formed through the extrusion of 300 ml of alginate inoculated with $0.5 * 10^6$ cells/ml_{alginate} through the 300 μ m nozzle. These capsules were then used to inoculate a minifors reactor containing 900 ml of media. The addition and removal of media in the perfusion culture began on day 5, when the concentration of the two key metabolites glucose and glutamine were below the concentration required by the exponentially growing cells that would be present in the reactor on the following day. Due to variations in the encapsulation procedure the average seeding viable cell density of the three batch encapsulated cultures and one perfusion culture performed was $0.32 * 10^6$ cells/ml_{reactor} +/- $0.165 * 10^6$ cells/ml. As the controlled-fed perfusion culture acted as a batch culture up until day 5, there should be no significant difference in the behaviour of the culture from days 0-5 in comparison with the 3 batch cultures conducted in Chapter 5. Figures 6.2.1.2.2.1, 6.2.1.2.2.1 and Table 6.2.1.2.2.1 allow for comparison to be made between the batch and perfusion cultures in regards to cell growth (cells/ml_{reactor}), cell viability, %, and growth rate and maximum viable cell densities achieved. Both cultures displayed an initial lag period of one day before entering exponential phase at the maximum growth rate from days 1-5. There was no significant difference in the growth rate of the cells in the batch and perfusion cultures from days 1-5 as expected. After cessation of exponential growth the viable cell density increased for one more day before the batch cultures entered a stationary phase for approximately 2 days. Following the stationary phase, a rapid fall off in cell viability on day 9 may be noted. In the controlled-fed perfusion culture, the initial perfusion of the culture on day 5, did not result in an increased viable cell density perml_{capsules} or perml_{reactor} than that which was observed in the batch cultures on day 6. However the perfusion strategy did come into play in that the cells/ml_{capsules} did continue to increase on days 7-8 in the perfusion culture, when the batch cultures were evidently in a stationary

phase. However evaluation of the $\text{cells/ml}_{\text{reactor}}$ for the perfusion culture would suggest that the cells were actually in stationary phase during days 7-8. This is however due to the fact that the number of capsules present in the perfusion reactor was decreasing daily from days 7 until the end of the culture on day 9. It is estimated that there were approximately 29 % less capsules present in the perfusion culture when viable cell density/ml capsules was at a maximum on day 8. Therefore although it was predicted that there was a 36 % increase in the maximum viable cell density ($\text{cells/ml}_{\text{capsules}}$), the failure of the capsules as a retention system (29 % loss of capsules) resulted in a reduced maximum viable cell density ($\text{cells/ml}_{\text{reactor}}$). Up to one third of the extracapsular cells resulting from broken capsules were removed from the reactor in the out flowing perfusion harvest stream. The extracapsular cell density of day 8 in the controlled-fed perfusion culture was $1.35 * 10^6$ cells/ml in comparison to $0.21 * 10^6$ cells/ml in the batch culture. (During perfusion and removal of harvest stream, conditions were not homogeneous as agitation was turned off so it was difficult to determine true extracapsular cell numbers. However the two figures calculated so indicate the failure of retention in perfusion).

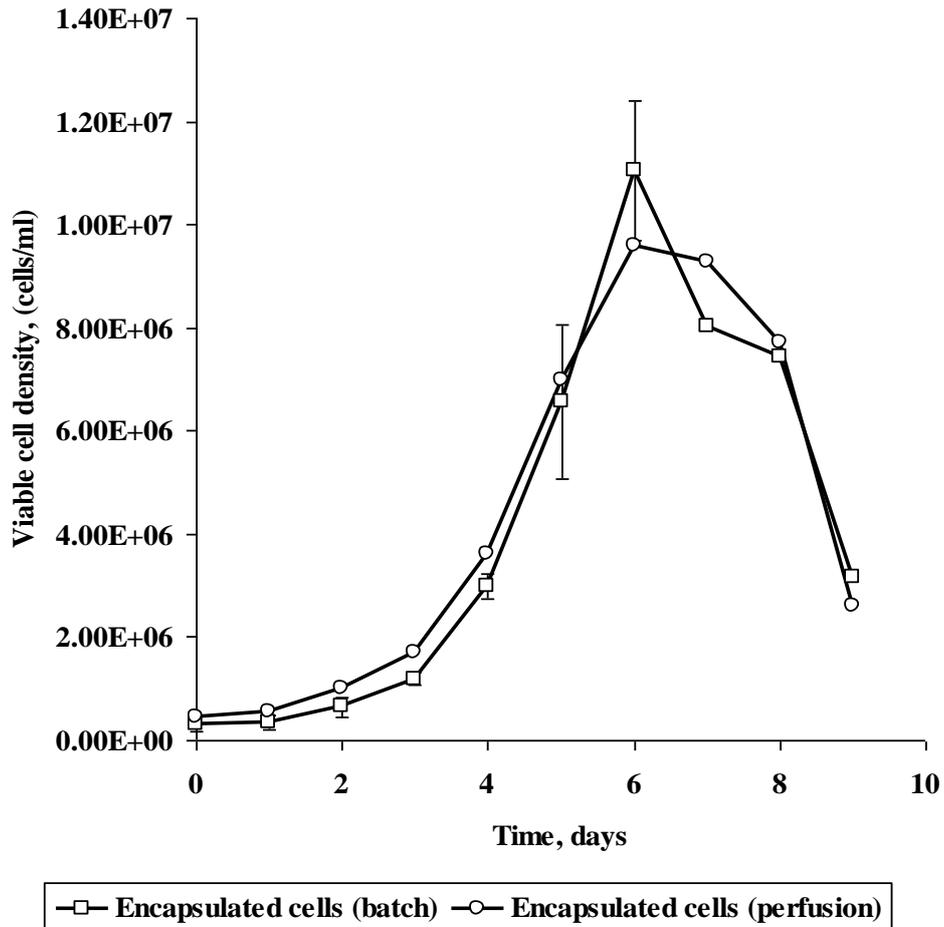


Figure 6.2.1.2.2.1: Growth of encapsulated CHO cells cultured in 300 μm liquid core APA microcapsules in a 1.7 L minifors reactor under batch and perfusion modes of cultivation. The cell concentration is given with respect to the volume of the reactor. Cell numbers were enumerated on a daily basis using trypan blue exclusion method and a Neubauer haemocytometer. Encapsulated cells were liberated prior to counting via extrusion of a capsule/PBS suspension through a 300 μm needle 3 times. Duplicate cell counts were performed. Values for the batch cultures are the average of triplicate cultures performed in parallel, +/- STD. Values for the perfusion culture are those which were obtained for the single culture carried out.

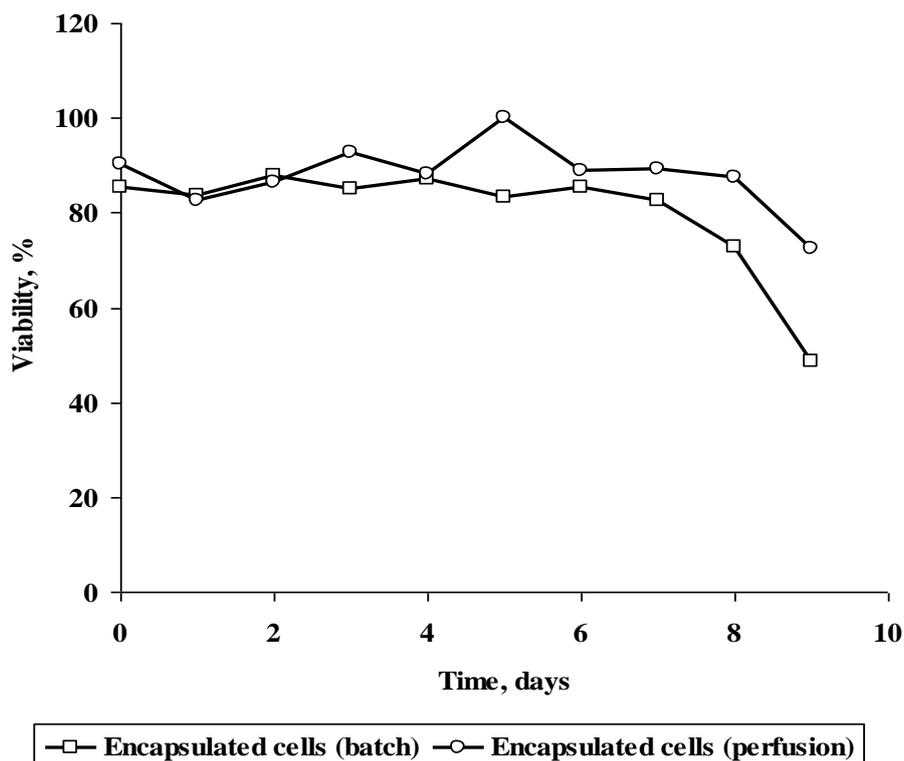


Figure 6.2.1.2.2.2: % Viability of CHO cells encapsulated in 300 µm liquid capsules and cultured under batch and perfusion modes of cultivation. % viability was determined by the enumeration of total and live cell numbers present in the culture using trypan blue exclusion method and Neubauer haemocytometer. Cell counts were performed in duplicate and average % viability determined on a daily basis. Values presented above are the average of triplicate cultures carried out in parallel +/- STD for the batch encapsulated cultures. Values for the perfusion culture are those obtained for the single culture performed.

Table 6.2.1.2.2.1: Calculated maximum specific growth rates displayed by cells encapsulated in 300 µm liquid capsules over the exponential growth period under batch and perfusion cultivation conditions. The growth rate was calculated from a plot of the logarithm of the viable cell density over the cultivation time (days). The maximum viable cell density is presented with respect to per ml reactor. The values presented below are the average of triplicate batch cultures performed in parallel, +/- STD. The values for the perfusion culture are those obtained for the single perfusion culture carried out.

Culture	μ_{max} (day ⁻¹)	Max VCD (*10 ⁶ cells/ml _{reactor})
Encapsulated cells (batch)	0.759 +/- 0.12	11.02 +/- 1.35
Encapsulated cells (perfusion)	0.636	9.6

6.2.1.3: Effect of perfusion mode on CHO cell productivity

To evaluate the effect of the perfusion mode of cultivation on rIgG1 production, media samples were removed from both the batch and perfusion cultures on a daily basis from day 5 onwards. As explained in Section 2.3.6., of the Materials and Methods Section, rIgG1 was purified from media samples removed from the cultures after centrifugation at 200 g for 5 minutes to remove any extracapsular cells. Capsules were separated from the media before centrifugation. rIgG1 present in the media was purified using Protein A Nab spin columns and quantified using the nanodrop at A 280 nm.

Figure 6.2.1.3.1 below allows comparison of the cumulative total amount of rIgG1 produced by cells cultivated under both batch and perfusion modes of cultivation in 300 μm liquid capsules. The values presented for the batch cultures are the mean of triplicate cultures +/- standard deviation. The amount of IgG1 produced by the encapsulated cells cultured in the perfusion reactor was not outside the standard deviation of the amount of IgG1 produced by the cells cultured in the batch reactor. Due to the fact that the $\text{cells/ml}_{\text{reactor}}$ was similar for both the batch and perfusion cultures, it would be expected that there would be no increase in the total IgG1 produced in the perfusion culture, providing that the specific productivity of the cells was not influenced. In order to ensure that the studies were comparable, the cell specific productivity was determined for both cultures before the perfusion strategy was applied. There was no difference in the specific production rate between the two modes of cultivation, as noted in Table 6.2.1.3.1. The fact that the volumetric productivity was the same over the entire culture period and there were essentially the same number of cells present in the cultures, this indicates that at later stages when $\text{cells/ml}_{\text{capsule}}$ were higher in perfusion, there were no changes in specific productivity.

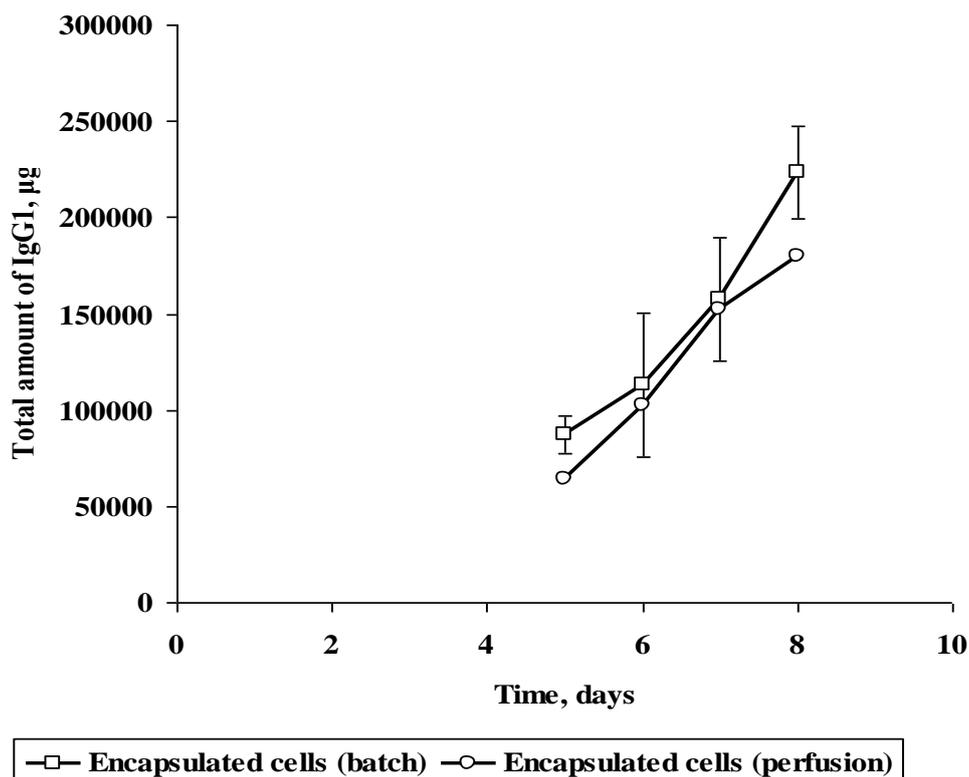


Figure 6.2.1.3.1: Total amount of IgG1 (μg) measured in cell free media samples harvested from encapsulated cultures on day 5 onwards. rIgG1 present in the media was purified using commercially available protein A/G Nab spin columns and quantified using the nanodrop A280 nm in duplicate which the average value recorded.. Values for cells cultured under batch conditions are the average of triplicate cultures performed in parallel, \pm STD. Values presented for perfusion conditions are those obtained for the single culture performed.

Table 6.2.1.3.1: Calculated rIgG1 production rates and cell specific production rates for cells encapsulated in 300 μm radius liquid capsules and cultured under batch and perfusion modes of cultivation. The specific production rate was calculated over the exponential growth phase for all cultures. The results presented below for the batch cultures are the average of triplicate cultures performed in parallel, \pm STD. The values for the perfusion culture are those obtained for the single culture performed under such conditions.

Culture	IgG1 production rate $\mu\text{g}/\text{day}$	q_{IgG1} $\mu\text{g}/10^6$ cells/day
Encapsulated cells (batch)	42274 \pm 398	2.39 \pm 1.02
Encapsulated cells (perfusion)	39637	2.28

6.2.1.4: Effect of perfusion mode on CHO cell metabolism

Both the batch and controlled-fed perfusion cultures were performed in ExCell CHO 325 PF media supplemented with an initial 4 mM L-glutamine concentration. The concentration of glucose in the commercially available media is expected to be 17.4 mM. The concentrations of the metabolites in the media were not altered during the batch cultures. In the case of the controlled-fed perfusion culture, after inoculation with 300 ml of capsules, the 1.7 L (1.2 L working volume) minifors reactor was operated for 5 days in batch mode. Perfusion was started on the 5th day. Offline glucose and glutamine assay results indicated that on day 5 the concentration of glucose and glutamine were below the level necessary to maintain the growth of the exponentially growing cells for the predicted number of cells expected to be present on the following day. Glucose and glutamine were monitored throughout the run as indicators of the concentration of the two metabolites to be included in the perfusion feed medium. The perfusion rate essentially remained constant. Lactate concentrations were later determined using high performance liquid chromatography (HPLC) as described in Materials and Methods Section 2.3.3.

The concentration of the major carbon sources, glutamine and glucose, and their corresponding by-products, ammonia and lactate, were measured on a daily basis (Figure 6.2.1.4.1 and 6.2.1.4.2). Determination of the concentration of the various metabolites enabled the calculations of the specific substrate uptake rate for each of the experimental cultures (Table 6.2.1.4.1).

Metabolic analysis of cells cultured under batch conditions was extensively evaluated in Chapter 5. By the end of the exponential growth phase all of the glutamine had been depleted. Glucose was depleted by the end of the stationary phase in batch cultures. As the perfusion mode of operation only began from on day 5 in the controlled-fed perfusion cultures, this culture acted essentially as a batch culture until this stage. It would therefore be assumed that all cell specific metabolite production and consumption rates would be the same during the cultivation period of days 1-5. Given that the cell number per ml_{reactor} was not significantly different between the batch and perfusion cultures from day 1-5, it would also be expected that the volumetric consumption and production rates would not be significantly different. Analysis of the cell specific and volumetric production and consumption rates, calculated during the exponential growth period, and presented in Table 6.2.1.4.1 below verify this assumption. There was no significant difference in the specific consumption rate displayed by the cells in the controlled-fed perfusion culture beyond day 5, where the value was

determined to be $3.98 * 10^{-4}$ mmoles/ 10^6 cells/day. The glucose concentration in the reactor was increased to 25 mM after perfusion on day 6 as it was assumed that the cells would continue to colonise the capsules. When it was noted that the cells/ml_{reactor} was not going to increase daily after day 6, due to capsule breakup and washout of cells from reactor, the concentration of glucose added to the perfusion media was adjusted accordingly. The specific lactate production rate was slightly higher after day 5, however it still fell within the standard deviation of the batch cultures at $6.26 * 10^{-4}$ mmoles/ 10^6 cells/day. Due to the slightly higher specific lactate production rate displayed by the cells, the concentration of lactate present at the end of the controlled-fed perfusion culture was 25 % higher than that observed in the batch culture. The overall yield of lactate on glucose in the controlled perfusion culture was similarly 27 % higher than that observed in the batch culture.

This may be attributed to the fact that the perfusion strategy design was based on the assumption that cells would continue growing exponentially. Therefore the concentration of glucose to be added to the culture each day was determined based on the assumption that the cells would continuously grow at the rate they were doing so in that time period. This however was not the case due to the instability of the capsules and a cell wash out during perfusion. The cells were essentially overfed resulting in high lactate yields.

Similarly for the metabolite glutamine and its corresponding by-product ammonia, there was no significant difference in both the cell specific and volumetric consumption and production rates between the batch cultures and the control-fed perfusion cultures from days 1-5. The specific glutamine and ammonia consumption and production rates remained similar to that observed during days 1-5 for the duration of the culture period at values of $7.29 * 10^{-5}$ and $7.67 * 10^{-5}$ mmoles/ 10^6 cells/day, respectively. The molar yield ratio of ammonia on glutamine was slightly lower than that observed for the batch culture over the entire culture period, but perhaps not significantly.

It must be noted that there is a difference in the initial concentration of L-glutamine between the batch and control-fed perfusion encapsulated cultures. Greater errors occur in the initial L-glutamine concentration in the media used for encapsulated cultures due to two main factors. Unlike glucose, L-glutamine is added to the media by the user prior to culturing, which introduces some batch-batch variations in the concentration between cultures due to human errors in volumes added. The media is also diluted by the microcapsules. Due to a combination of variations in the volume of microcapsules made per batch and the amount of washing buffer remaining in the vessel with the prepared microcapsules prior to addition of

the media, variations in initial concentration of nutrients can occur between cultures. The culture media becomes diluted by both the microcapsules and any of the washing buffer remaining.

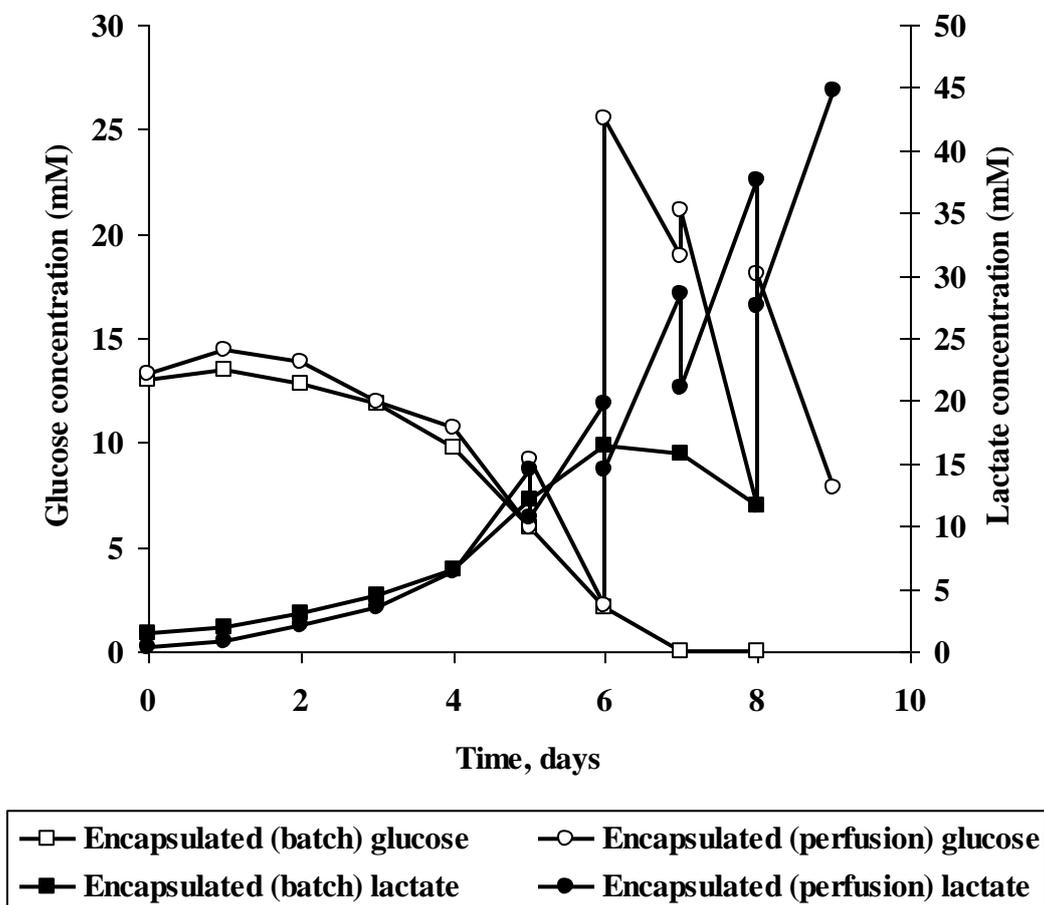
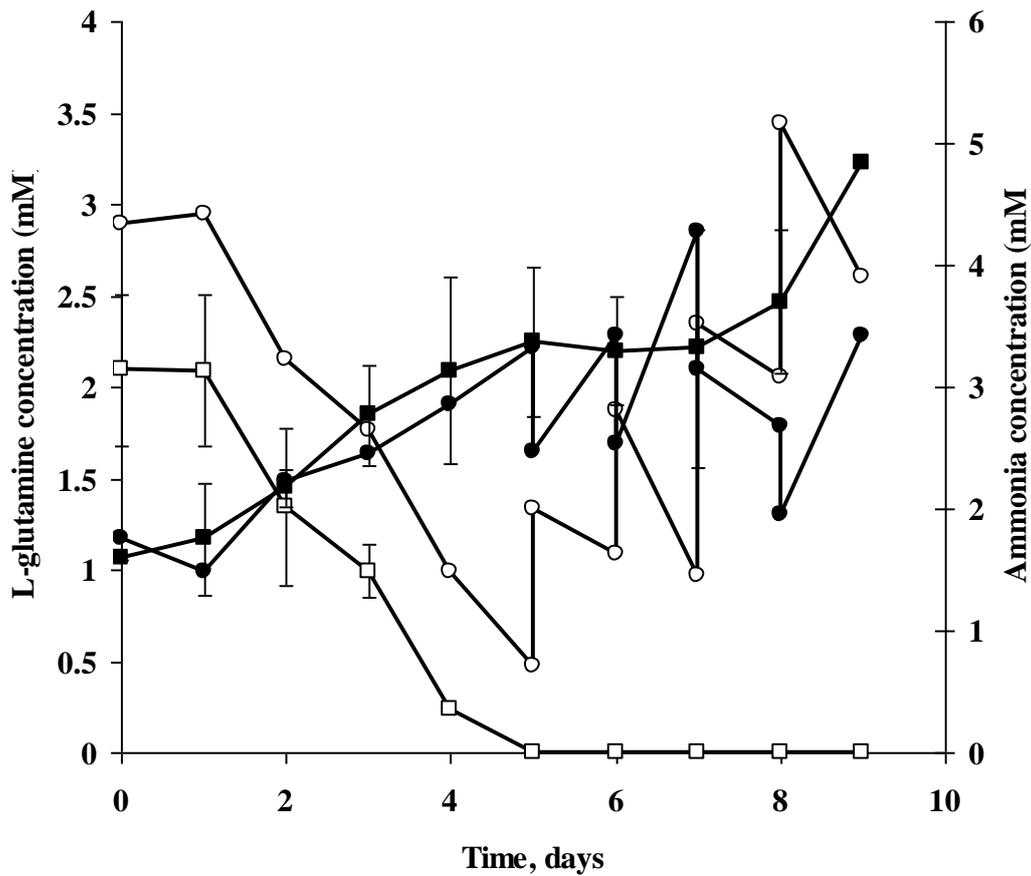


Figure 6.2.1.4.1: Concentration of glucose and lactate present in both batch and perfusion cultures over the duration of the respective cultivation periods. The concentrations of glucose were determined using an enzymatic assay kit and lactate concentrations were determined offline by HPLC analysis using Supelco H column. Batch values are the average of triplicate cultures carried out in parallel, +/- STD. The values for the perfusion culture are those which were obtained for the single culture performed.



—□— Encapsulated (batch) glutamine —○— Encapsulated (perfusion) glutamine
 —■— Encapsulated (batch) ammonia —●— Encapsulated (perfusion) ammonia

Figure 6.2.1.4.2 Concentration of glutamine and ammonia present in both batch and perfusion cultures over the duration of the respective cultivation period. The concentrations of glutamine and ammonia were determined offline using a commercial available enzymatic assay kit. Batch values are the average of triplicate cultures carried out in parallel, +/- STD. The values for the perfusion culture are those which were obtained for the single culture performed.

Table 6.2.1.4.1: Calculated rates of consumption and production of key metabolites (glucose and lactate) as determined by offline analysis throughout the culture period. Cell specific consumption and production rates of the key metabolites were determined during the exponential growth phase (days 1-5). Yields were calculated over the entire cultivation period. Batch values are the average of those calculated for triplicate cultures carried out in parallel, +/- STD. The values for the perfusion culture are those which were calculated for the single culture performed.

	Encapsulated batch	Encapsulated perfusion
Glucose consumption rate (mM/day)	2.09 +/- 0.12	2.026
q_{glucose} (mmoles/ 10^6 cells/day)	$3.11 * 10^{-4} +/- 1.12 * 10^{-4}$	$3.90 * 10^{-4}$
Lactate production rate (mM/day)	2.55 +/- 0.71	3.15
q_{lactate} (mmoles/ 10^6 cells/day)	$5.32 * 10^{-4} +/- 1.88 * 10^{-4}$	$5.93 * 10^{-4}$
$Y_{\text{lactate/glucose}}$ (mmole/mmole)	1.22 +/- 0.18	1.55

Table 6.2.1.4.2: Calculated rates of consumption and production of key metabolites (glutamine and ammonia) as determined by offline analysis throughout the culture period. Cell specific consumption and production rates of the key metabolites were determined during the exponential growth phase (days 1-5). Yields were calculated over the entire cultivation period. Batch values are the average of those calculated for triplicate cultures carried out in parallel, +/- STD. The values for the perfusion culture are those which were calculated for the single culture performed.

	Encapsulated batch	Encapsulated perfusion
Glutamine consumption rate (mM/day)	0.59 +/- 0.19	0.62
$q_{\text{glutamine}}$ (mmoles/ 10^6 cells/day)	$8.42 * 10^{-5} +/- 6.21 * 10^{-6}$	$7.89 * 10^{-5}$
Ammonia production rate (mM/day)	0.459 +/- 0.180	0.387
q_{ammonia} (mmoles/ 10^6 cells/day)	$1.31 * 10^{-4} +/- 1.16 * 10^{-5}$	$8.36 * 10^{-5}$
$Y_{\text{ammonia/glutamine}}$ (mmole/mmole)	1.88 +/- 0.23	1.53

6.2.1.5: Effect of perfusion mode on product quality

To assess the effect of a perfusion strategy as a mode of cultivation on the quality of rIgG1 produced, both glycan and aggregation analysis were completed on the purified rIgG1 samples harvested from both the batch and controlled-fed perfusion encapsulated cultures at both the end of the exponential and stationary growth phases. Samples harvested from the cultures were subjected to N-glycan release and labelled as outline in Materials and Methods Section 2.3.7.1. Hydrophilic interaction liquid chromatography (HILIC) characterisation of the released and labelled N-glycans produced a chromatogram consisting of 8 distinctly separated peaks. Each of the peaks was assigned a distinctive glucose units (GU) value by comparison to the retention times of the standard A-AB labelled external dextran. Examination of exoglycosidase treated samples allowed for the corresponding number and linkage of the component monosaccharide units represented by each GU value to be elucidated, as described in Materials and Methods section 2.3.7.3. Aggregation analysis of the purified samples was carried out using size exclusion chromatography (SEC)

Figure 6.2.1.5.1 illustrates the relative % of glycans present on rIgG1 harvested from the batch and perfusion encapsulated cultures on day 5 of the culture period (i.e. before the perfusion strategy was applied). No significant difference may be noted in the relative % of each type of glycan present on the rIgG1 harvested on day 5 of the batch and perfusion encapsulated cultures. As the perfusion culture essentially acted as a batch culture until day 5, it would be expected that there would be no significant differences in the relative % of each glycan present on the protein at this stage. Irrelevant of the mode of cultivation, all cultures displayed a similar trend in the relative % of glycans present. The earliest form of N-glycosylation of rIgG1 is denoted by M5, F6A1/A2 and F6A2. The highest relative percentage of glycans present on the rIgG1 samples were the early glycoforms, predominantly F6A2. The galactosylated glycoforms are denoted by F(6)A2[6]G(4)1 and F(6)A2[3]G(4)1, which contain 1 galactose unit on either of the outer GlcNAc arms, and F(6)A2G2, which contains a galactose residue on each of the outer GlcNAc arms. Of the galactosylated glycoforms present, the F(6)A2[6]G(4)1 were the predominant form in all cultures, independent of the mode of cultivation employed.

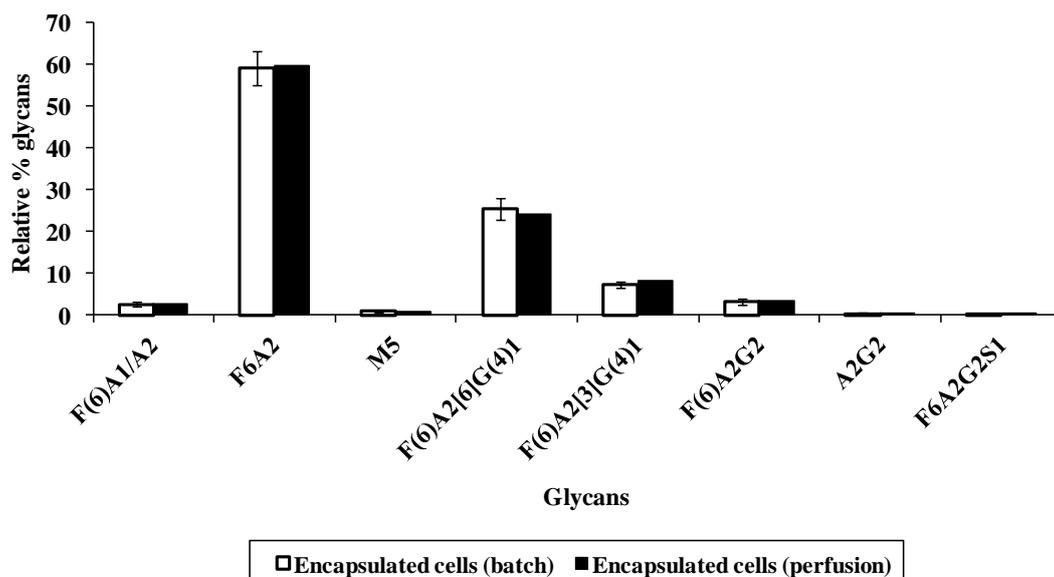


Figure 6.2.1.5.1 : Relative % of N-glycan forms detected on rIgG1 samples harvested from batch and perfusion encapsulated cultures on day 5 of the culture period (before perfusion was initiated) N-glycans were released from the rIgG1 samples by means of PNGaseF digestion. Samples for HILIC analysis were labeled by adding 5 μ l of 2AB labelling solution. The retention time of the glycan peaks was compared with an external A-AB labelled dextran standard for structural elucidation by converting the retention time of the peaks into GU. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out in parallel for the batch cultures, +/- STD. Values for the perfusion culture are those which were obtained for the single culture carried out.

In Chapter 5 there was a noted lower relative % of M5 present on the rIgG1 protein harvested from encapsulated cultures in comparison to suspension cultures, at the end of the exponential growth period. There was however an increase in the relative % of M5 present on IgG1 throughout the culture period for the encapsulated culture. The increase in the relative % of M5 present on IgG1 in encapsulated cultures was larger than that which was observed for suspension culture and so by the end of the stationary growth phase the relative % M5 present on IgG1 was higher than that observed in stationary culture.

In this current study, M5 did also increase over the cultivation period of the controlled-fed perfusion culture. However the increases in the relative % of M5 in the perfusion culture were less dramatic than those noted in the batch cultures, **Table 6.2.1.5.1.**

Table 6.2.1.5.1: Relative % of M5 present on rIgG1 harvested from batch and control-fed perfusion cultures on days 5-7 as detected using HILIC following PNGaseF release and 2-AB labelling. Each IgG1 sample was analysed in duplicate.

Time	Relative % M5 Batch	Relative % M5 Perfusion
Day 5	0.94	0.91
Day 6	1.24	0.97
Day 7	2.71	1.2

A 2.8- fold increase in the relative % of M5 glycans was noted over the duration of the stationary phase in the batch cultures. However only a 32 % increase in the relative % of M5 was noted in the perfusion cultures

By the end of the stationary phase (day 8 controlled-fed perfusion), there was 38 % less M5 present on protein harvested from perfusion cultures, in comparison to batch cultures.

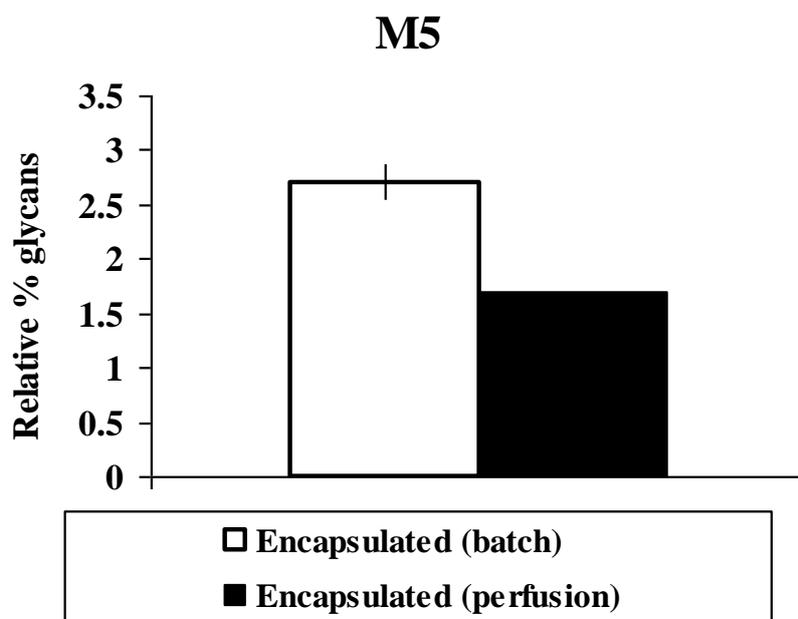


Figure 6.2.1.5.2: Relative % of M5 present on rIgG1 harvested from batch encapsulated cultures and controlled perfusion cultures at the end of the stationary growth phases (day 7 batch and day 8 controlled-fed perfusion) as detected using HILIC following PNGaseF release and 2-AB labelling. Each IgG1 sample was analysed in duplicate. The values presented for the batch are an average of triplicate cultures performed in parallel, +/- STD. The values for the perfusion culture are those which were obtained for the single culture performed.

Another important observation that can be made is that there was an increase in the level of fucosylated, sialyated glycans between the exponential and stationary phases in the controlled-fed perfusion culture. Due to the fact that the perfusion culture was not performed in triplicate, it is difficult to confirm if the finding is significant.

Table 6.2.1.5.2: Relative % of F(6)A2G2S1 present on rIgG1 harvested from batch and control-fed perfusion cultures at the end of the exponential and stationary growth phases as detected using HILIC following PNGaseF release and 2-AB labelling. Each IgG1 sample was analysed in duplicate. The values presented for the batch are an average of triplicate cultures performed in parallel, +/- STD. The values for the perfusion culture are those which were obtained for the single culture performed.

Time	Relative % F6A2G2S1 batch	Relative % F6A2G2S1 perfusion
End exponential phase	0.24 +/- 0.04	0.18
End stationary phase	0.3 +/- 0.03	0.34

Looking at the information provided in Table 6.3.1.5.2, it may be observed that there was an 80 % increase in the relative % of F(6)A2G2S1 present on IgG1 harvested at the end of the stationary phase in comparison to the end of the exponential phase for the control-fed perfusion culture. However it may also be noted that the relative % of the glycan present in the controlled-fed perfusion is simply at the lower range of levels found in the batch culture for the same time period. Also, the relative % of the glycan present in the controlled-fed perfusion culture is simply at the higher range of the levels found in the batch cultures.

Over 95 % of the rIgG1 harvested from culture media was in a monomeric form for both culture conditions tested, Figure 6.2.1.6.3

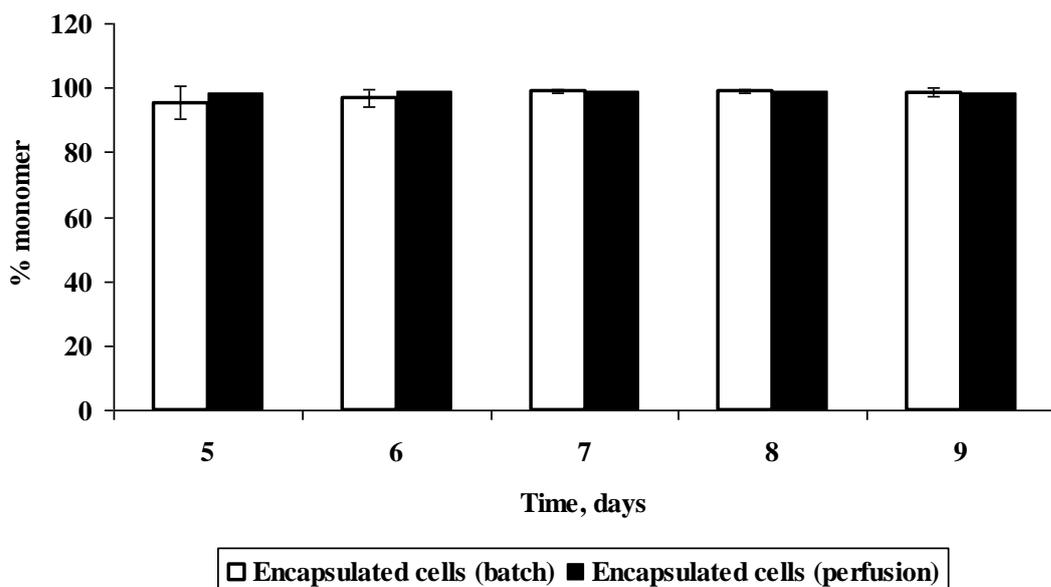


Figure 6.2.1.5.3: Relative % monomeric form of rIgG1 samples as detected by SEC. IgG1 samples were harvested from cultures batch and perfusion cultures performed. IgG1 samples were analysed in duplicate. The results are presented as the average of triplicate cultures performed for the batch encapsulated cultures, +/- STD. Values for the perfusion cultures are those which were obtained for the single culture performed.

6.2.2: Batch encapsulated –v- Perfusion encapsulated in 1 L Erlenmeyer shake flasks

Based on the results obtained from section 6.2.1, it may be concluded that further colonisation of the capsules was achieved by operating a controlled-fed perfusion cultivation strategy. The attainment of a higher cell density/ml_{reactor} was however limited due to the breakup of capsules in the harsh bioreactor environment. A decision was therefore made to repeat the controlled-fed perfusion experiment, however at a smaller scale, in the less harsh 1 L Erlenmeyer shake flask environment.

The results of Chapter 4 suggested that, when in suspension, the CHO cells behave differently when cultured in shake flasks and 1.7 L minifors reactors platforms. Such differences by noted by: reduced growth rate of 11 %, reduced specific productivity of 28 %, increased lactate production from glucose and reduced glycosylation in notably the levels of galactosylation for suspension cells cultivated in 1.7 L minifors reactor platform in comparison to a 1 L Erlenmeyer shake flask platform.

Therefore, before the perfusion culture may be completed in the shake flask, it is necessary to observe if there are similar differences in cellular activity noted between the cultivation of

encapsulated cells in shake flask and minifors reactor platforms. For this reason batch encapsulated shake flask cultures were performed for comparison to the batch encapsulated cultures previously carried out in the 1.7 L minifors reactor . Controlled-fed perfusion cultivated encapsulated cells in 1 L Erlenmeyer shake flasks are not directly comparable to batch cultivated encapsulated cells in the 1.7 L minifors reactor as changes in cellular activity may be brought about by the change in culture vessel as oppose to the change in cultivation mode. This experiment will ensure that any alterations that may be noted in encapsulated cellular activities under a controlled-fed perfusion mode of cultivation in shake flasks (section 6.3.3) are in fact due to the controlled-fed perfusion mode of cultivation and not the scale down

6.2.2.1: Capsule characterisation

6.2.2.1.1: Capsule size

In both the 1.7 L minifors reactor cultures and 1 L Erlenmeyer shake flask cultures, capsules were prepared by the extrusion of alginate through the 300 μm nozzle used in all experiments to date. Capsule size was determined on a daily basis. Thirty capsules were measured individually in order to calculate the average diameter and the standard deviation. Generally the size distribution was in the range of 3-5 %.

Figure 6.2.2.1.1 below illustrates the capsules radius size over the cultivation period for both the batch cultures. The results presented below are the average of triplicate cultures. Capsule size throughout the 1 L Erlenmeyer shake flask culture is consistent with that observed during the batch cultures. A greater size standard deviation is noted between the triplicate cultures performed in the 1.7 L minifors reactor culture. This may be attributed to the larger volume of capsules required per run (300 ml) in comparison to that required for shake flask cultures (100 ml).

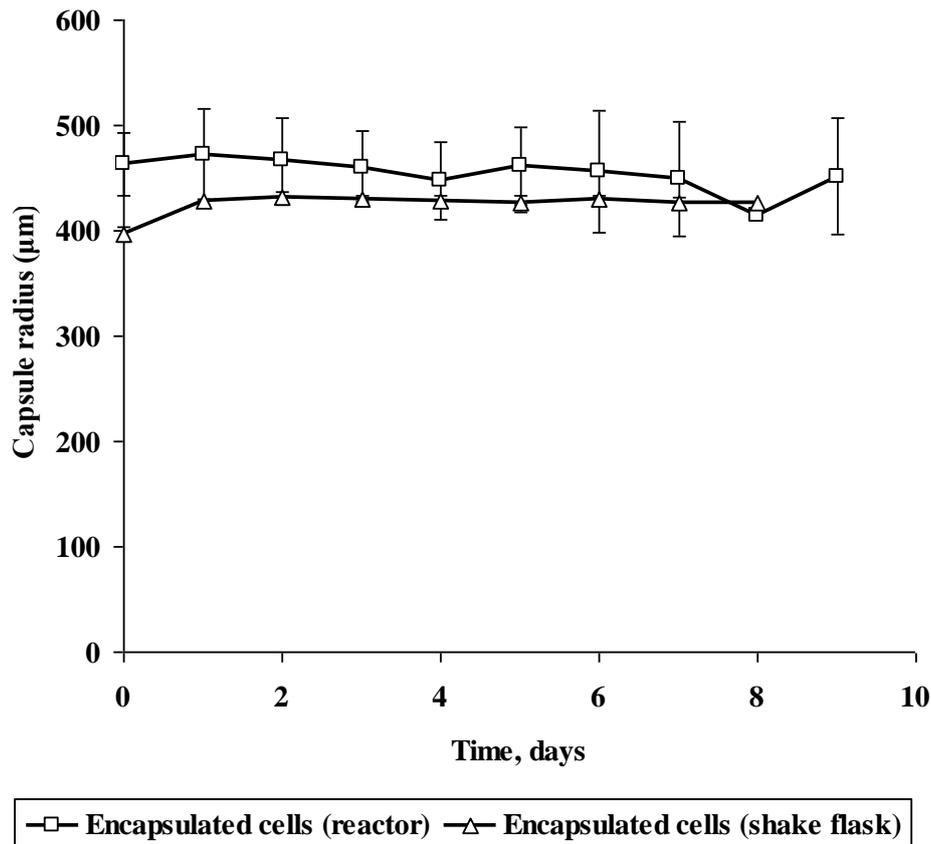


Figure 6.2.2.1.1.1: Average capsule radius size (μm) \pm STD over the duration of the cultivation period for each of the three encapsulated cultures completed in both 1.7 L minifors reactor and 1 L Erlenmeyer shake flask platforms. The size of approximately 30 capsules was measured at each time point and an average determined.

6.2.2.1.2: Capsule strength

The mechanical strength of the microcapsules in the batch cultures conducted in both 1.7 L minifors reactor and 1 L Erlenmeyer shake flask platforms was routinely evaluated in terms of Force (g)/capsule which the capsule could withstand using the Texture analyser.

Figure 6.2.2.1.2.1 below illustrates the mechanical resistance of the microcapsules in both 1.7 L minifors reactor and 1 L Erlenmeyer shake flask platforms over the cultivation period. For both culture platforms there is a dramatic decrease in the mechanical resistance of the capsules from days 0-3, after which the resistance is noted to fall slower before somewhat levelling off. On day 3 the mechanical resistance of microcapsules in the 1.7 L minifors reactor was determined to be $\sim 0.07 \pm 0.01$ g/capsule, as noted in Chapter 5. However it is not until day 5 in the 1 L Erlenmeyer shake flask models that the capsule reach such a low

resistance. It is interesting to note that the profile of mechanical resistance in both culture platforms are typically the same. The aim of this work was to identify if the shake flask model could be beneficial in reducing the potential for capsule break up which was noted to occur later in perfusion cultures performed above in 1.7 L minifors reactor cultures. Evaluation of the results below would suggest that the shake flask environment offers no increased benefits for maintaining capsule resistance at a higher level for the entire culture period than that observed in the 1.7 L minifors reactor cultures. The decrease in mechanical resistance is however reduced. It may be hypothesised that the shake flask environment may allow these capsule with low mechanical resistance ($\sim 0.07\text{-}0.08$ g/capsule) to remain intact due to the exposure to less harsh shearing forces than would be observed in the minifors reactor. Due to the batch nature of the cultures, the cultures were terminated due to a lack of cell viability. Therefore whether the capsules resist breakage in shake flasks for extended period of time in comparison to reactor cultures under control-fed perfusion strategies in shake flasks will have to be determined.

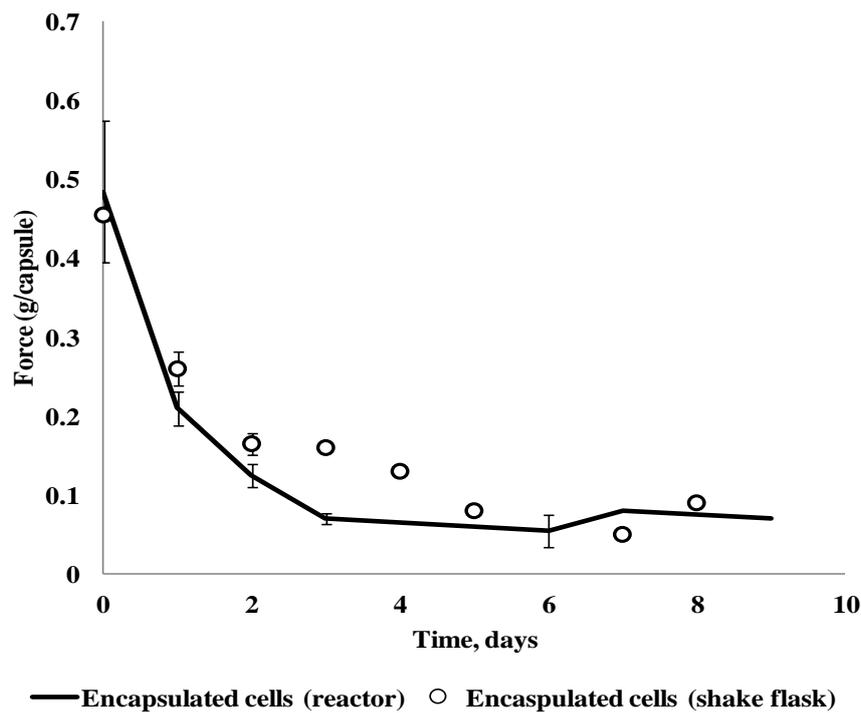


Figure 6.2.2.1.2.1: Mechanical resistance (force (g)/capsule) over cultivation period for 300 μm APA microcapsules containing CHO DP-12 cells cultivated in a 1.7 L minifors reactor and 1 L Erlenmeyer shake flask platforms under batch cultivation conditions. A total of 3 layers of microcapsules were analysed on a daily basis. Values presented are average of triplicate analysis \pm STD for one of the encapsulated cultures carried out in the study.

6.2.2.2: Comparability of encapsulated cell growth under batch conditions in 1 L Erlenmeyer shake flasks and 1.7 L minifors reactors.

In order to test the comparability of encapsulated CHO DP-12 cells grown in 1 L Erlenmeyer shake flasks, with that previously obtained for encapsulated cells cultured in 1.7 L minifors reactors, 100 ml of CHO DP-12 containing APA microcapsules were used to inoculate 300 ml of culture medium in 1 L Erlenmeyer shake flasks. Both 1.7 L reactor cultures and 1 L Erlenmeyer shake flask cultures were conducted in triplicate. The capsule to media ratio of volume was kept constant between the two culture platforms. For the minifors reactor culture the bioreactor was operated under conditions of 37 °C and 100 rpm agitation and 95 % DO by means of sparging with compressed air (flowrate of 0.01 vvm). The pH was also controlled at 7.2 through automatic addition of 2 M NaOH, via a feed line, or CO₂ sparging (through the headspace at a flowrate of 0.047 vvm). Shake flasks were incubated in 37 °C incubator in unvented shake flasks and 100 rpm agitation. All conditions were consistent with those utilised in Chapter 4 in which a comparison study was conducted between the growth of suspension cells in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. The average seeding density was $0.9 * 10^6$ (cells/ml_{capsules}).

Figure 6.2.2.2.1 below demonstrates the growth of the encapsulated CHO DP-12 cells inside the microcapsules in both the shake flask and reactor platforms. The values are expressed at cells/ml_{capsules}. It is evident from the data portrayed that there was no significant difference in the growth pattern or maximum cell number reached in the capsules between the two culture platforms investigated. The main steps forward from this study are to progress towards applying the controlled-fed perfusion strategy to the 1 L Erlenmeyer shake flask encapsulated cultures in a similar means to that which was completed for the 1.7 L minifors reactor cultures in section 6.2.1. Therefore it is important to identify at this early stage if the 1 L Erlenmeyer shake flask platform has the potential to allow the capsules to act as a suitable retention system for the application of controlled-fed perfusion strategy. Figure 6.2.2.2.2 below demonstrates the growth of CHO DP-12 cells cultured in both 300 µm APA microcapsules and freely in suspension in both 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. The average viable cell densities in this case are expressed as cells/ml_{vessel}. The data for the suspension cultures is taken from Chapter 4 in order to enable visualisation of the progress made to date in achieving high cell density cultures. Addressing the possibility of capsule retention in 1 L Erlenmeyer shake flask cultures, it is understood that there is no significant difference in the growth pattern displayed by encapsulated cells

when cultured in 1L Erlenmeyer shake flask and 1.7 L minifors reactor platforms in $\text{cells/ml}_{\text{vessel}}$. The retention capabilities of the capsules were not compromised by the transition to 1 L Erlenmeyer shake flask platforms for the encapsulated cultures. This observation is also strengthened by the similar extracapsular cell concentration observed in the 1 L Erlenmeyer shake flask study and the 1.7 L minifors reactor study were the same ($0.21 * 10^6$ cells/ml)

It is important to note that when batch encapsulated cultures were initially conducted in 1.7 L minifors reactor cultures, no cause for concern arose as to the suitability of the APA microcapsules as retention devices suitable for an extended cultivation period during a controlled-fed perfusion culture. Caution must therefore be taken to the fact that when the controlled-fed perfusion strategy may be applied, capsule breakup, similar to that noted in section 6.2.1 may still occur. In regards to the progression of the thesis towards high cell density culture systems, the return to a shake flask model with the encapsulated cells has not resulted in any advancements in cellular growth rate or maximum viable cell densities than those achieved through the encapsulation of the cells and cultivation in 1.7 L minifors reactor platforms, as discussed in Chapter 5. The increases in maximum viable cell densities achieved for batch encapsulated cultures in comparison to batch suspension cultures, in the 1.7 L minifors reactor platform have been maintained in the encapsulated 1 L Erlenmeyer shake flask cultures, **Table 6.2.2.2.1**.

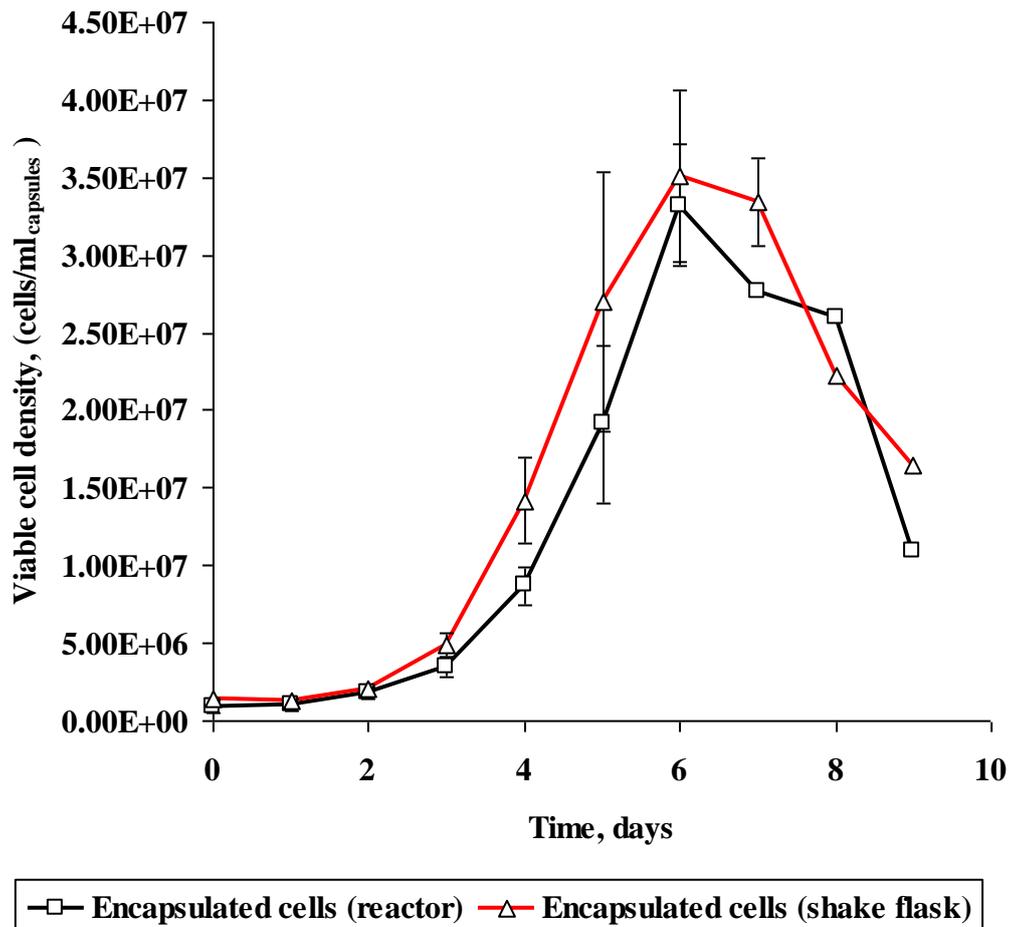


Figure 6.2.2.2.1 Growth of CHO cells cultured in 300 µm liquid core APA microcapsules under batch cultivation conditions in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. Cell numbers were enumerated on a daily basis using trypan blue exclusion method and a Neubauer haemocytometer. Encapsulated cells were liberated prior to counting via extrusion of a capsule/PBS suspension through a 300 µm needle 3 times. Duplicate cell counts were performed. The cell concentration is given with respect to the capsule volume. Values for both platforms are the average (+/- STD) of triplicate cultures.

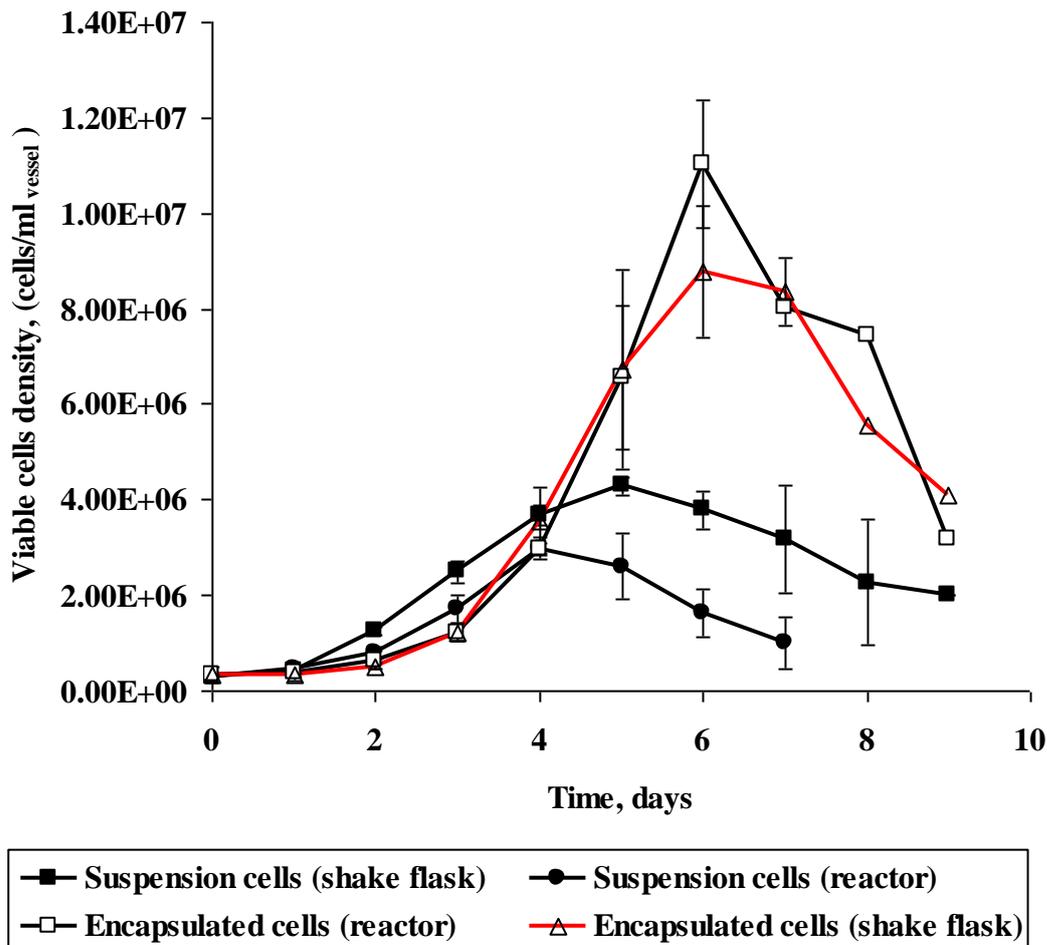


Figure 6.2.2.2.2: Growth of CHO DP-12 cells both encapsulated in 300 μm APA microcapsules and freely in suspension under batch modes of cultivation in 1L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. Cell numbers were enumerated on a daily basis using trypan blue exclusion method and a Neubauer haemocytometer. Encapsulated cells were liberated prior to counting via extrusion of a capsule/PBS suspension through a 300 μm needle 3 times. Duplicate cell counts were performed. The cell concentration is given with respect to the vessel working volume which the cultures were performed in. Values for both platforms are the average (+/- STD) of triplicate cultures.

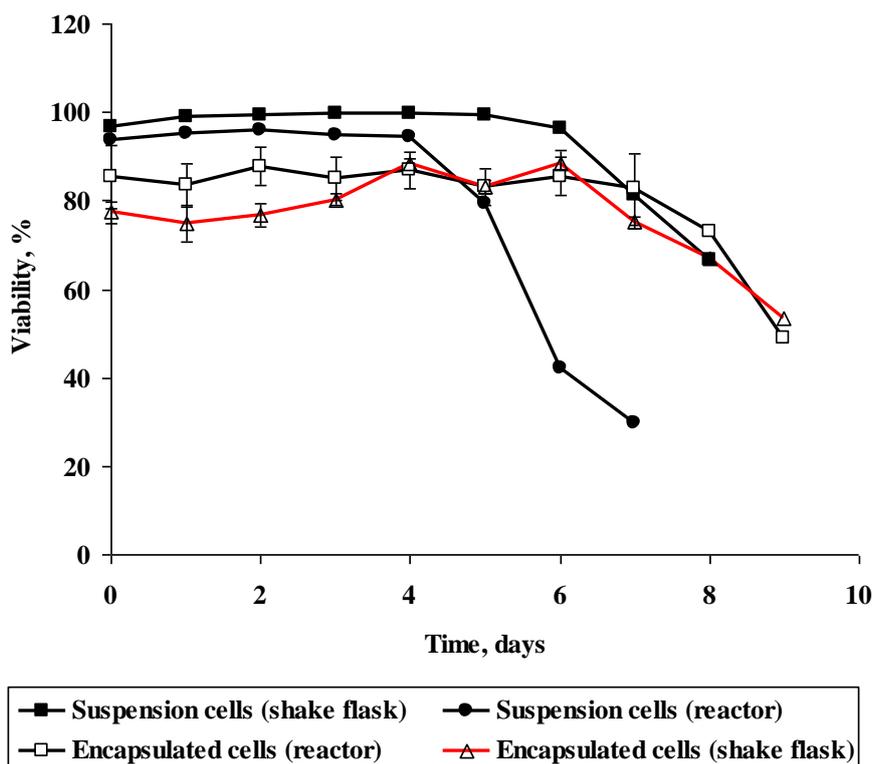


Figure 6.2.2.2.3: % Viability of CHO cells cultured in suspension and encapsulated in 300 μm liquid capsules in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor cultures. % viability was determined by the enumeration of total and live cell numbers present in the culture using trypan blue exclusion method and Neubauer haemocytometer. % viability is expressed as a percentage of live cells over dead cells present. Cell counts were performed in duplicate and average % viability determined on a daily basis. Values are average (+/- STD) of triplicate cultures performed in parallel at both culture platforms.

Table 6.2.2.2.1: Calculated maximum specific growth rates displayed by cells cultured in suspension and encapsulated in 300 μm liquid capsules in 1L Erlenmeyer shake flask and 1.7 L minifors reactor platforms over the exponential growth period. The growth rate was calculated from a plot of the logarithm of the viable cell density over the cultivation time (days). The maximum viable cell density is presented with respect to per $\text{ml}_{\text{vessel}}$. Values are average (+/- STD) of triplicate cultures performed in parallel at both culture platforms.

Culture	μ_{max} (day^{-1})	Max VCD ($*10^6$ cells/ $\text{ml}_{\text{vessel}}$)
Suspension cells (shake flask)	0.725 +/- 0.013	4.30 +/- 0.19
Suspension cells (reactor)	0.645 +/- 0.037	3.12 +/- 0.27
Encapsulated cells (reactor)	0.759 +/- 0.13	11 +/- 1.35
Encapsulated cells (shake flask)	0.789 +/- 0.06	9.21 +/- 1.15

6.2.2.3: Comparability of encapsulated cell metabolism under batch conditions in 1 L Erlenmeyer shake flasks and 1.7 L minifors reactors.

Both the 1 L Erlenmeyer shake flask and 1.7 L minifors reactor encapsulated cultures were performed in ExCell CHO 325 PF media supplemented with an initial 4 mM L-glutamine concentration. The concentration of glucose in the commercially available media is expected to be 17.4 mM. The concentration of the major carbon sources, glutamine and glucose, and their corresponding by-products, ammonia and lactate, were measured from media samples taken from all cultures on a daily basis. Samples were removed from cultures on a daily basis and centrifuged at 200 g for 5 minutes, followed by filtration of the supernatant (0.22 μm PTFE) in order to remove cells and culture debris. As stated above glutamine and ammonia analysis was carried out using commercially available enzymatic assays kits. Glucose and lactate concentrations were later determined using HPLC as described in Materials and Methods Section 2.3.3. Determination of the concentration of the various metabolites enabled the calculations of the specific substrate uptake and production rate for each of the experimental cultures (Table 6.2.2.3.1-6.2.2.3.3).

The results from suspension cultures in 1.7 L minifors reactor and 1 L Erlenmeyer shake flasks (Chapter 4) are also presented in the tables in order to demonstrated how the movement back to the 1L Erlenmeyer shake flask platform has in no way compromised the progress to date regarding the achievement of high density cultures brought about by encapsulation.

By the end of the exponential growth phase all of the glutamine had been depleted in both the encapsulated 1 L Erlenmeyer shake flask and 1.7 L minifors reactor cultures. Glucose was depleted by the end of the stationary phase in such cultures. There was no significant difference in the glucose consumption rate (mM/day) displayed by the encapsulated cells when cultured in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. Both were lower than that observed for the suspension cultures conducted in the 1.7 L minifors reactor as discussed in Chapter 5. The batch encapsulated 1L Erlenmeyer shake flask culture also displayed a lower glucose consumption rate (mM/day) than the batch suspension 1L Erlenmeyer shake flask culture noted to be 3.20 ± 0.003 in Chapter 4. This again verifies the conclusion from Chapter 5 in which it was deduced that encapsulation alters the metabolism of cells in comparison to conditions in which they are cultured in suspension, thus allowing encapsulated cells to have lower glucose consumption rates (mM/h). The specific glucose consumption rates (mmoles/ 10^6 cells/day) were not significantly different for

the two encapsulated cultures. The specific consumption rates were therefore again lower than the suspension reactor and suspension shake flask cultures.

The lactate production rate (mM/day) and the specific lactate production rate (mmoles/ 10^6 cells/day) were not significantly different between the batch encapsulated 1L Erlenmeyer shake flask and batch encapsulated 1.7 L minifors reactor cultures. An unfavourably high lactate production rate was noted for the suspension cultures in the 1.7 L minifors reactor culture in comparison to all other cultures. This is verified by the increases specific production rate for this culture. In agreement with the results presented in Chapter 5, the lactate production rate was also lower for the batch encapsulated 1 L Erlenmeyer shake flask cultures, in comparison to the batch suspension 1 L Erlenmeyer shake flask cultures.

Both the glutamine consumption rate (mM/day) and cell specific consumption rate (mmoles/ 10^6 cells/day) were not significantly different when encapsulated cells were cultured in both 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. As noted in Chapter 5, there was a 73 % decrease in the specific glutamine consumption for the encapsulated cells cultured in the 1.7 L minifors reactor, in comparison to the suspension cells cultured in the same environment. These results are verified in this study by comparison of the specific glutamine consumption rate displayed by the encapsulated cells cultured in the 1 L Erlenmeyer shake flask platform to the suspension cells cultured under the same conditions. A notably 65 % decrease in the specific consumption rate was noted for the encapsulated cells, verifying the fact that encapsulation of cells favours lower specific glutamine consumption.

No significant difference was noted in the ammonia production rate (mM/day) for encapsulated cells cultured in the 1 L Erlenmeyer shake and 1.7 L minifors reactor platform. The cell specific rate (mmoles/ 10^6 cells/day) was determined to be slightly lower in the 1 L Erlenmeyer shake flask culture in comparison to the 1.7 L minifors reactor culture. However there are significantly large standard deviations for each of the calculated figures. It should also be noted that both figures are significantly lower than those calculated for the suspension cultures in both culture platforms.

As all specific rates of glucose and lactate consumption and production were similar to that displayed by encapsulated cells cultured in both 1 L Erlenmeyer shake flask cultures and 1.7 L minifors reactor cultures, as expected there was no significant difference in the yield of lactate from glucose in these cultures. The yield of ammonia on glutamine was 35 % lower for encapsulated cells cultured in shake flasks in comparison to that observed for

encapsulated cells cultured in 1.7 L minifors reactor. This may be attributed to the lower specific ammonia production rate displayed by the cells cultured in 1 L Erlenmeyer shake flasks. The mean maximum concentration of ammonia present in the encapsulated cultures performed in 1 L Erlenmeyer shake flasks was 3.68 +/- 0.09 mM. The corresponding mean maximum concentration of ammonia present in the encapsulated cultures performed in the 1.7 L minifors reactor cultures was 31 % higher at 4.83 +/- 0.60 mM.

Table 6.2.2.3.1: Calculated volumetric and specific consumption and production rates of glucose and lactate in batch suspension and encapsulated cultures conducted on 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. Values are average (+/- STD) of those calculated for triplicate cultures performed in parallel at both culture platforms.

Culture	Glucose consumption rate (mM/day)	Lactate production rate (mM/day)	q_{glucose} (mmoles/10 ⁶ cells/ml)	q_{lactate} (mmoles/10 ⁶ cells/ml)
Suspension cells (shake flask)	3.20 +/- 0.03	3.49 +/- 0.17	$8.3 * 10^{-4}$ +/- $5.23 * 10^{-5}$	$1.15 * 10^{-3}$ +/- $8.59 * 10^{-5}$
Suspension cells (reactor)	5.38 +/- 0.39	10.55 +/- 0.40	$1.69 * 10^{-3}$ +/- $1 * 10^{-4}$	$3.1 * 10^{-3}$ +/- $3.33 * 10^{-4}$
Encapsulated cells (reactor)	2.09 +/- 0.11	2.55 +/- 0.71	$3.11 * 10^{-4}$ +/- $1.12 * 10^{-4}$	$5.33 * 10^{-4}$ +/- $1.9 * 10^{-4}$
Encapsulated cells (shake flask)	1.89 +/- 0.06	1.99 +/- 0.18	$2.69 * 10^{-4}$ +/- $7.87 * 10^{-5}$	$3.42 * 10^{-4}$ +/- $8.24 * 10^{-5}$

Table 6.2.2.3.2: Calculated volumetric and specific consumption and production rates of glutamine and ammonia in batch suspension and encapsulated cultures conducted on 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. Values are average (+/- STD) of those calculated for triplicate cultures performed in parallel at both culture platforms.

Culture	Glutamine consumption rate (mM/day)	Ammonia production rate (mM/day)	$q_{\text{glutamine}}$ (mmoles/10 ⁶ cells/ml)	q_{ammonia} (mmoles/10 ⁶ cells/ml)
Suspension cells (shake flask)	0.89	0.902	$2.23 * 10^{-4}$	$3.11 * 10^{-4}$
Suspension cells (reactor)	0.81 +/- 0.06	0.78 +/- 0.04	$3 * 10^{-4}$ +/- $3.31 * 10^{-5}$	$3.46 * 10^{-4}$ +/- $2.74 * 10^{-5}$
Encapsulated cells (reactor)	0.59 +/- 0.19	0.46 +/- 0.18	$8.42 * 10^{-5}$ +/- $6.21 * 10^{-6}$	$1.31 * 10^{-4}$ +/- $1.16 * 10^{-5}$
Encapsulated cells (shake flask)	0.53 +/- 0.06	0.46 +/- 0.01	$7.81 * 10^{-5}$ +/- $2.97 * 10^{-5}$	$5.98 * 10^{-5}$ +/- $1.98 * 10^{-5}$

Table 6.2.2.3.3: Calculated yields of byproduct/metabolite (mmoles/mmoles) over the cultivation period for both encapsulated and suspension cultures conducted using 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. Values are average (+/- STD) of those calculated for triplicate cultures performed in parallel at both culture platforms.

Culture	Y_{lactate/glucose} (mmoles/mmoles)	Y_{ammonia/glutamine} (mmoles/mmoles)
Suspension cells (shake flask)	0.95 +/- 0.11	1.07
Suspension cells (reactor)	1.81 +/- 0.09	1.23 +/- 0.15
Encapsulated cells (reactor)	1.22 +/- 0.18	1.88 +/- 0.23
Encapsulated cells (shake flask)	1.01 +/- 0.05	1.22 +/- 0.11

6.2.2.4: Comparability of encapsulated cell productivity under batch conditions in 1 L Erlenmeyer shake flasks and 1.7 L minifors reactors.

To evaluate the effect of the two modes of cultivation on rIgG1 production in encapsulated cells, media samples were removed from both the 1 L Erlenmeyer shake flask culture and the 1.7 L minifors reactor cultures on a daily basis from day 5 onwards. As explained in Section 2.3.6., of the Materials and Methods Section, rIgG1 was purified from media samples removed from the cultures after centrifugation at 200 g for 5 minutes to remove cells. Capsules were separated from the media before the centrifugation step for the encapsulated culture samples. rIgG1 present in the media was purified using Protein A Nab spin columns and quantified using the nanodrop at A 280 nm.

Figure 6.2.2.4.1 below represents the concentration of IgG1 present in each of the suspension and encapsulated cultures conducted in both 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms at the end of the stationary phase of growth. The aim of the work is to identify if there are significant differences in the productivities of the encapsulated cells when cultured in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. No significant differences were observed in the cell specific productivity ($\mu\text{g}/10^6$ cells/day) or concentration of IgG1 at the end of the stationary phase in both the cultivation environments. In Chapter 5 it was concluded that the encapsulation of cells did not influence the specific productivity of the cells when compared to that displayed by suspension cells cultivated under the same conditions in the 1.7 L minifors reactor culture. Increases in the volumetric productivity in

this case were noted for the encapsulated cells due to an increase in the number of cells producing the recombinant protein. However a comparison of the productivity results for the encapsulated and suspension cells cultured in the 1 L Erlenmeyer shake flask platform do somewhat contradict this conclusion. The concentration of IgG present at the end of the stationary phase for the encapsulated cells is similar to that obtained when the cells were cultured in suspension in the same 1 L Erlenmeyer shake flask model. There were however a 2.5- fold increase in the number of cells producing the protein in the encapsulated cultures. The specific productivity of the suspension cells in the 1 L Erlenmeyer shake flask platform is ~ 2- fold higher than that observed for encapsulated cells cultured in the same platform, Table 6.2.2.4.1

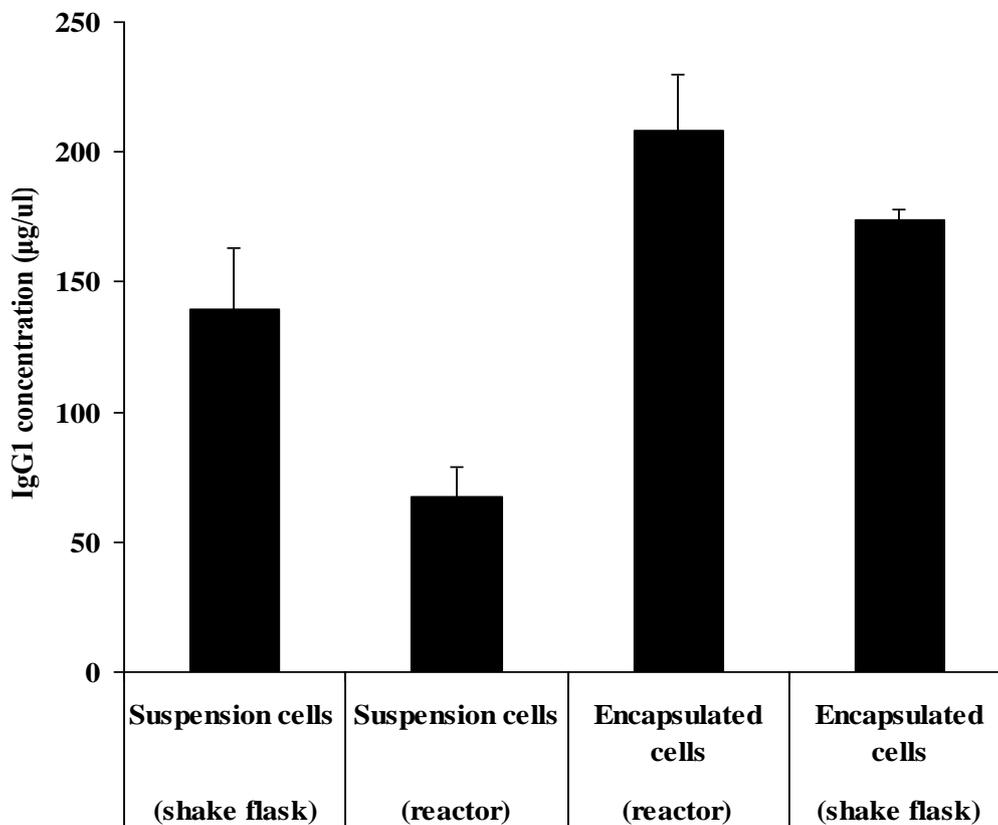


Figure 6.2.2.4.1: Concentration of rIgG1 measured in cell free media samples harvested from encapsulated and suspension cultures conducted in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor cultures. rIgG1 present in the media was purified using commercially available protein A/G Nab spin columns and quantified using the nanodrop A280 nm in duplicate which the average value recorded. Values are the average (+/- STD) of triplicate cultures performed in parallel for each of the conditions tested.

Table 6.2.2.4.1: Calculated rIgG1 production rates and cell specific production rates for cells cultured in suspension and encapsulated in 300 μm radius liquid capsules in both 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. The specific production rate was calculated over the exponential growth phase for all cultures. Values are the average (+/- STD) of triplicate cultures performed in parallel for each of the conditions tested.

Culture	IgG1 production rate ($\mu\text{g/ml/day}$)	q_{IgG1} ($\mu\text{g}/10^6$ cells/day)
Suspension cells (shake flask)	16.787	5.16 +/- 0.29
Suspension cells (reactor)	10.522	3.74 +/- 0.23
Encapsulated cells (reactor)	50.78 +/- 5.38	2.39 +/- 1.02
Encapsulated cells (shake flask)	39.54 +/- 10.82	2.75 +/- 0.67

6.2.2.5: Comparability of encapsulated cell product quality under batch conditions in 1 L Erlenmeyer shake flasks and 1.7 L minifors reactors.

To assess the comparability of the quality of IgG1 purified from encapsulated cultures performed in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms, both glycan and aggregation analysis were completed on the purified rIgG1 samples harvested from cultures at both the end of the exponential and stationary growth phases. Samples harvested from the cultures were subjected to N-glycan release and labelled as outline in Materials and Methods Section 2.3.7.1. HILIC characterisation of the released and labelled N-glycans produced a chromatogram consisting of 8 distinctly separated peaks. Each of the peaks was assigned a distinctive GU value by comparison to the retentions times of the standard A-AB labelled external dextran. Examination of exoglycosidase treated samples allowed for the corresponding number and linkage of the component monosaccharide units represented by each GU value to be elucidated, as described in Materials and Methods section 2.3.7.3. Aggregation analysis of the purified samples was carried out using SEC.

The overall aim of conducting glycan analysis on IgG1 samples from batch encapsulated 1 L Erlenmeyer shake flask cultures is to identify if there are any significant differences in the quality of the protein between that and the protein harvested from 1.7 L minifors encapsulated cultures.

However before such a comparison may be made, it is firstly necessary to investigate if any changes in the particular glycoform profile for the recombinant protein occurs over the duration of the culture period for the encapsulated 1 L Erlenmeyer shake flask culture. In summary, previous suspension cultures in both shake flasks and minifors reactors resulted in a glycoform profile which was consistent throughout the culture period (Chapter 4). For encapsulated cultures conducted in a 1.7 L minifors reactor platform, the relative % of the

glycan M5 was noted to significantly increase over the stationary growth period. For the batch encapsulated shake flask cultures the relative % of M5 did also increase over the course of the stationary growth phase, along with changes in 4 other glycans typically found on IgG1 including; the early unprocessed glycan F6A2, the fucosylated and galactosylated glycans F(6)A2[6]G(4)1 and F(6)A2G2 and the galactosylated glycan A2G2S1. The changes in the relative % of each of these glycans are displayed in Figures 6.2.2.5.1-6.2.3.5.5. M5 was noted to increase by 51 % over the course of the stationary phase for the encapsulated shake flask cultures. There was a 12 % decrease in the relative % of unprocessed F(6)A2 and a 42 % and 22 % increase respectively in the relative % of the fucosylated and galactosylated F(6)A2[6]G(4)1 and F(6)A2G2 glycans. The fully galactosylated but not fucosylated glycan A2G2S1 increased by 22 % from the end of the exponential growth phase until the end of the stationary phase.

All of the changes noted, with the exception of M5 are positive changes as they denote an increase in the degree of complexity of the glycan processing throughout the culture period. As the complexity of the protein is increasing, the alterations in the relative % of the glycans is not due to the action of glycosidase enzymes which may be released from cells should the viability decline during the stationary growth period.

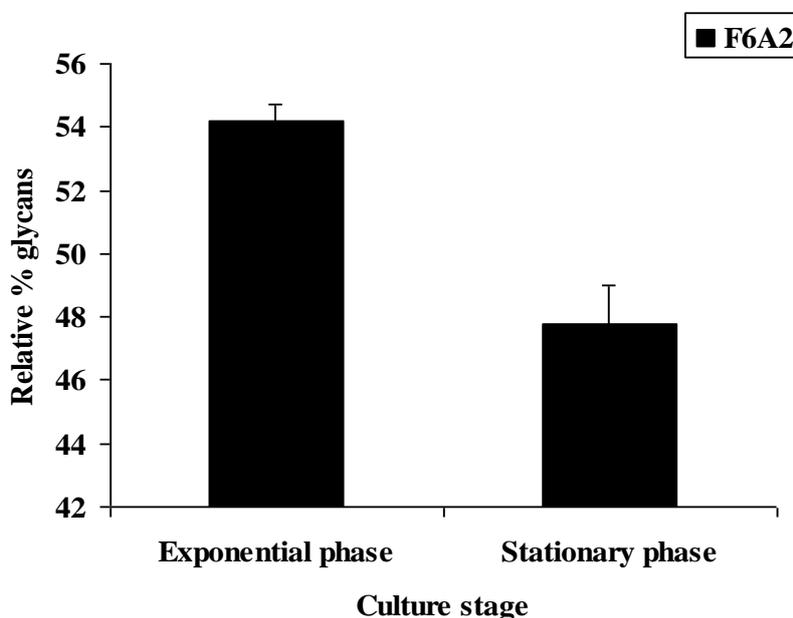


Figure 6.2.2.5.1: Relative % of F6A2 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flasks at the end of the exponential and stationary growth phases as detected using HILIC following PNGaseF release and 2-AB labelling. The results are the average of the triplicate cultures carried out in parallel +/- STD.

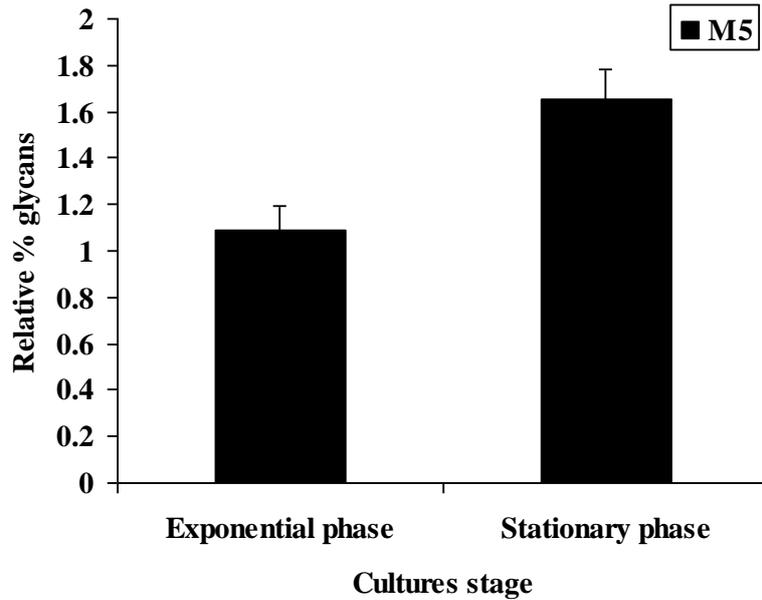


Figure 6.2.2.5.2: Relative % of M5 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flasks at the end of the exponential and stationary growth phases as detected using HILIC following PNGaseF release and 2-AB labelling. The results are the average of the triplicate cultures carried out in parallel +/- STD.

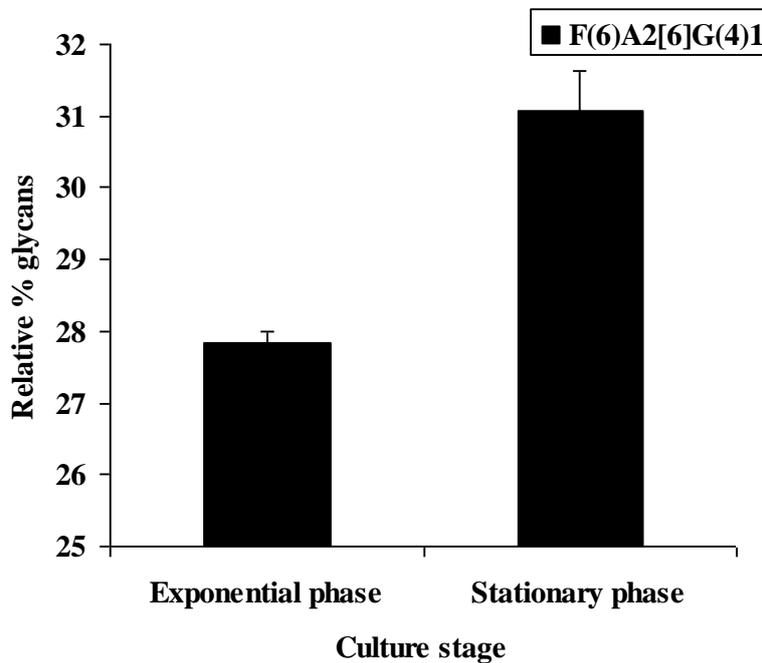


Figure 6.2.2.5.3: Relative % of F(6)A2[6]G(4)1 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flasks at the end of the exponential and stationary growth phases as detected using HILIC following PNGaseF release and 2-AB labelling. The results are the average of the triplicate cultures carried out in parallel +/- STD.

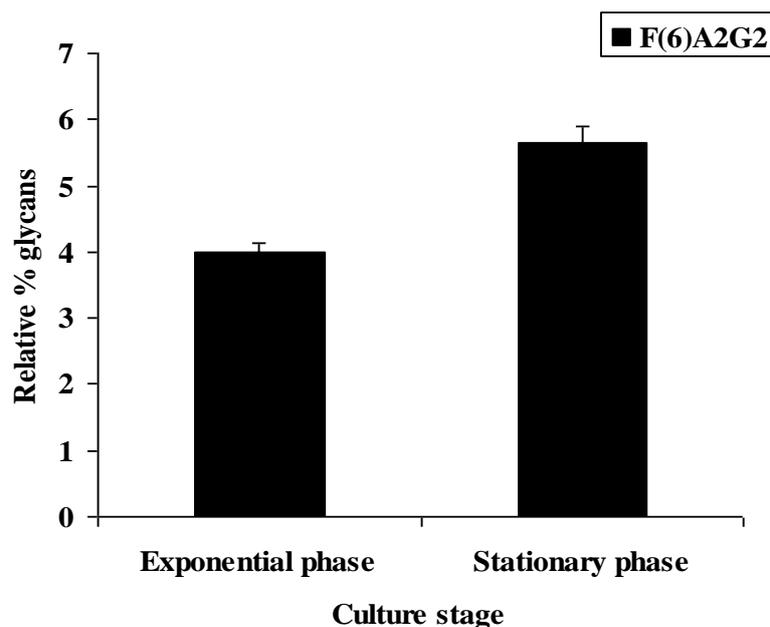


Figure 6.2.2.5.4: Relative % of F(6)A2G2 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flasks at the end of the exponential and stationary growth phases as detected using HILIC following PNGaseF release and 2-AB labelling. The results are the average of the triplicate cultures carried out in parallel +/- STD.

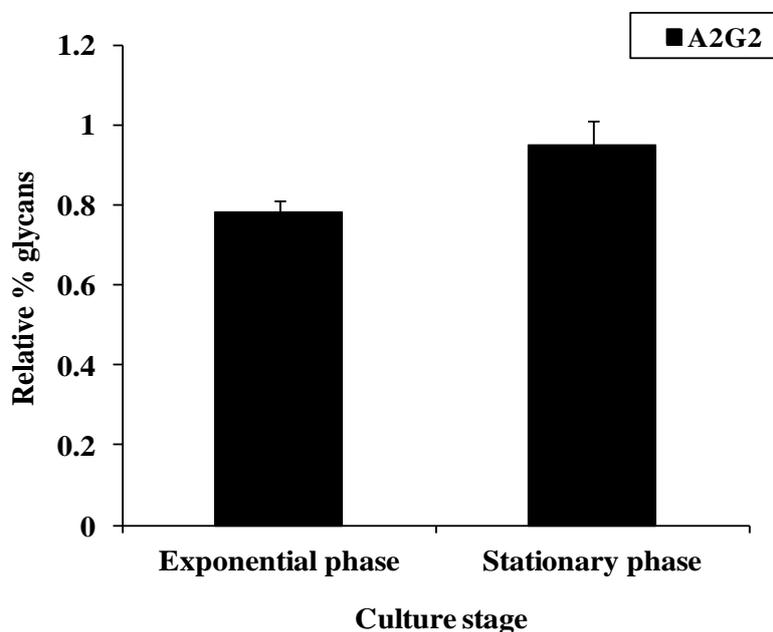


Figure 6.2.2.5.5: Relative % of A2G2 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flasks at the end of the exponential and stationary growth phases as detected using HILIC following PNGaseF release and 2-AB labelling. The results are the average of the triplicate cultures carried out in parallel +/- STD.

The main aim of this particular study is to note if there are any differences in the quality of the protein harvested from encapsulated cultures in 1L Erlenmeyer shake flask and 1.7 L minifors reactor culture platforms. Irrelevant of the mode of cultivation, all cultures displayed a similar trend in the relative % of glycans present. The earliest form of N-glycosylation of rIgG1 is denoted by M5, F6A1/A2 and F6A2. The highest relative percentage of glycans present on the rIgG1 samples were the early glycoforms, predominantly F6A2. The galactosylated glycoforms are denoted by F(6)A2[6]G(4)1 and F(6)A2[3]G(4)1, which contain 1 galactose unit on either of the outer GlcNAc arms, and F(6)A2G2, which contains a galactose residue on each of the outer GlcNAc arms. Of the galactosylated glycoforms present, the F(6)A2[6]G(4)1 were the predominant form in all cultures, independent of the mode of cultivation employed, Figure 6.2.2.5.6

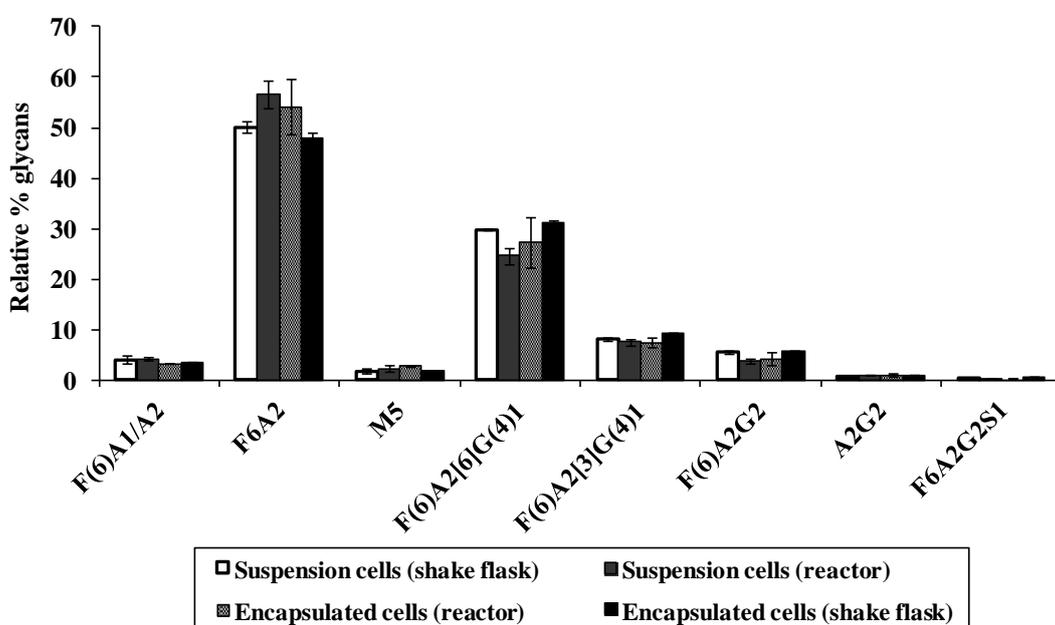


Figure 6.2.2.5.6: Relative % of N-glycan forms detected on rIgG1 samples harvested from batch suspension and encapsulated cultures performed in both 1 L Erlenmeyer shake flask and 1.7 L minifors reactor models. N-glycans were released from the rIgG1 samples by means of PNGaseF digestion. Samples for HILIC analysis were labeled by adding 5 μ l of 2AB labelling solution. The retention time of the glycan peaks was compared with an external A-AB labelled dextran standard for structural elucidation by converting the retention time of the peaks into GU. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for each condition tested, +/- STD.

The main changes however noted in the relative % of individual glycans present on IgG1 harvested from encapsulated 1 L Erlenmeyer shake flask and 1.7 L minifors reactor cultures were in M5 and F(6)A2G2S1. In both cultures the relative % of M5 was similar at the end of the exponential phase, the value for which increased by the end of the stationary phase. However the increase in M5 in the 1.7 L minifors reactor culture was much more dramatic than that which was noted for the 1 L Erlenmeyer shake flask culture, with an overall 39 % lower relative % of the glycan present in samples taken from the shake flask culture. The relative % of the fully fucosylated and fully sialyated glycan was 53% higher and the end of the exponential phase and 63% higher at the end of the stationary phase in batch encapsulated shake flask cultures in comparison to batch encapsulated reactor cultures, Figure 6.3.2.5.7. This is an interesting development as sialylation had not previously been noted to be different between batch encapsulated 1.7 minifors reactor cultures and batch suspension 1.7 L minifors reactor 1 L Erlenmeyer shake flask cultures.

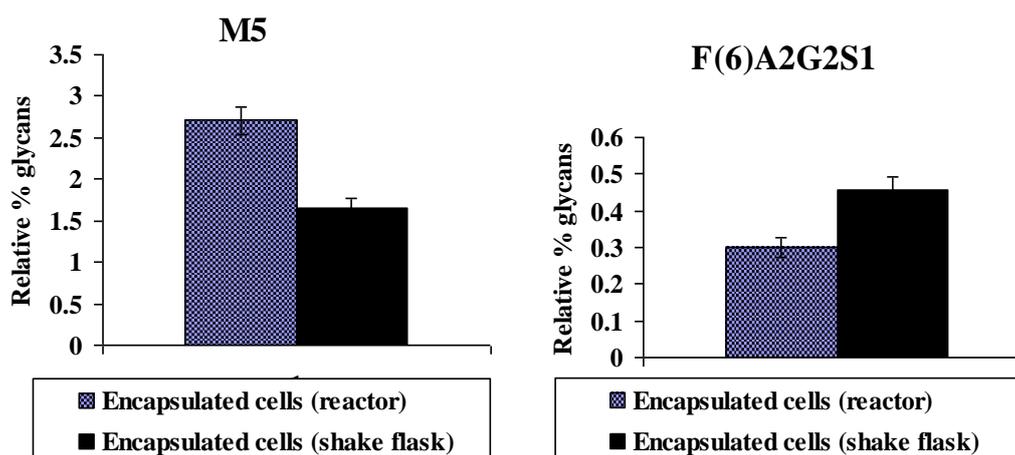


Figure 6.2.2.5.7: Relative % of M5 and F(6)A2G2S1 present on rIgG1 harvested from batch encapsulated cultures in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor cultures at the end of the stationary growth phase as detected using HILIC following PNGaseF release and 2-AB labelling. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for the two culture conditions tested +/- STD.

Although this section is aimed at outlining differences in protein quality between samples harvested from in batch encapsulated 1.7 L minifors reactor and 1 L Erlenmeyer shake flask cultures, attention may also be paid to the fact that the cultivation of encapsulated cells in the 1 L Erlenmeyer shake flask resulted in a 2-fold increase in F(6)A2G2S1 in comparison to that which was observed for suspension cultures under the same conditions.

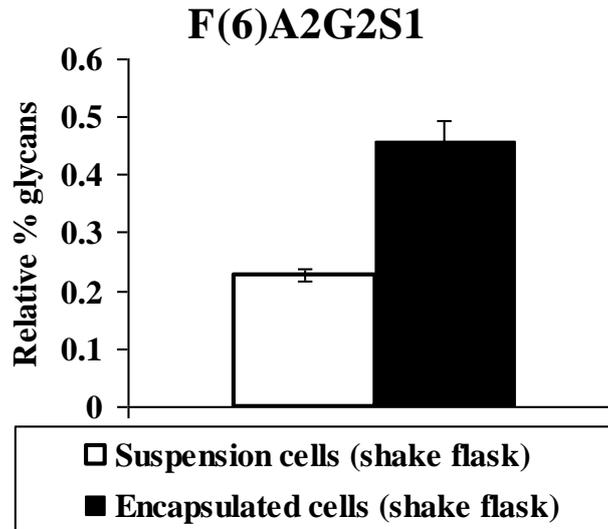


Figure 6.2.2.5.8: Relative % of F(6)A2G2S1 present on rIgG1 harvested from batch encapsulated and suspension cultures in 1 L Erlenmeyer shake flask at the end of the stationary growth phase as detected using HILIC following PNGaseF release and 2-AB labelling. Each IgG1 sample was analysed in duplicate. The results are the average of the triplicate cultures carried out for the two culture conditions tested +/- STD.

Over 95 % of the protein harvested for both conditions tested was of monomeric form.

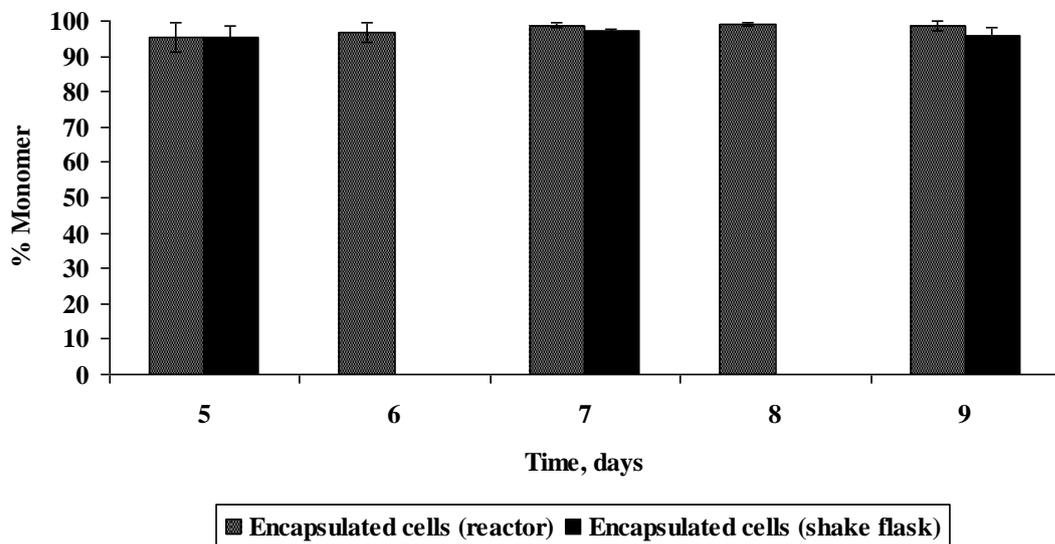


Figure 6.2.2.5.9: Relative % monomeric form of rIgG1 samples as detected by SEC. IgG1 samples were harvested from encapsulated cultures performed in 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. IgG1 samples were analysed in duplicate. The results are presented as the average of triplicate cultures performed for the two culture conditions tested +/- STD.

6.2.3: Cultivation of encapsulated cells in shake flasks under batch (non-CO₂ incubator), perfusion (non-CO₂ incubator) and perfusion (CO₂ incubator) cultivation conditions

In an effort to investigate the possibility for enhanced colonisation (>5 %) in comparison to that which was observed when cells were cultured under batch conditions in both 1.7 L minifors reactor platforms and 1 L Erlenmeyer shake flask platforms, a controlled perfusion strategy has been applied to encapsulated cells culture in 1 L Erlenmeyer shake flasks. The study has been conducted in both the standard unvented flasks in a non-CO₂ incubator and in vented flasks in a 5 % CO₂ incubator in order to overcome any mass transfer issues in the levels of dissolved gases. The basis for using the 1 L Erlenmeyer shake flask platform is as a direct result of the findings obtained in Section 6.3.1 in which the stability of the microcapsules and their suitability as a retention device in a perfusion culture became extremely compromised in the 1.7 L minifors reactor, noted by a 58 % decrease in the total number of capsules present in the reactor between days 7-9. Under batch cultivation conditions, the 1 L Erlenmeyer shake flask culture did extend to a cultivation time of 9 days, after which cell viability was limited by the batch nature of the applied cultivation mode. It is predicted that due to the less harsh environment that the 1 L Erlenmeyer shake flask provides, in comparison to the 1.7 L minifors reactor, an extended study on the influence of the controlled-fed perfusion strategy on capsule colonisation may be achieved. The aim was to further enhance the colonisation of the capsules beyond that which has been achieved in Section 6.3.1 (~9 %). Any alterations in cellular activities brought about by the controlled-fed perfusion strategy or enhanced colonisation were also investigated.

Flasks were seeded with 100 ml of APA microcapsules containing cells at a density of 0.5×10^6 cells/ml_{alginate} in 300 ml of ExCell CHO 325 PF media. In keeping inline with the study in section 6.2.1, the cultures were initiated with the application of a constant perfusion rate. 100 ml of spent medium was removed and replaced with 100 ml of fresh medium on a daily basis for the entire cultivation period for flasks in the non-CO₂ incubator. For flasks cultured in the 5 % CO₂ incubator, two of such medium changes were made per day in order to reduce the concentration of ammonia in the cultures. Based on offline analysis of cell numbers and metabolite concentrations (glucose and glutamine), forward rate adjustments were made in the concentration of the nutrients based on predicted cell numbers for next day and their nutrient requirement. All cultures were conducted in triplicate.

6.2.3.1: Capsules characterisation

6.2.3.1.1: Capsules size

In the 1 L Erlenmeyer shake flask cultures, capsules were prepared by the extrusion of alginate through the 300 μm nozzle used in all experiments to date. Capsule size was determined on a daily basis. Thirty capsules were measured individually in order to calculate the average diameter and the standard deviation. Generally the size distribution was in the range of 3-5 %.

Figure 6.2.3.1.1.1 below illustrates the capsules radius size over the cultivation period for the batch cultures, control-fed perfusion (non-CO₂) and control-fed perfusion (CO₂). The results presented below are the average of triplicate cultures. Capsule size throughout the 1 L Erlenmeyer shake flask cultures have a relatively low standard deviation between each other indicating no alterations in capsule size as a result of the culture conditions applied.

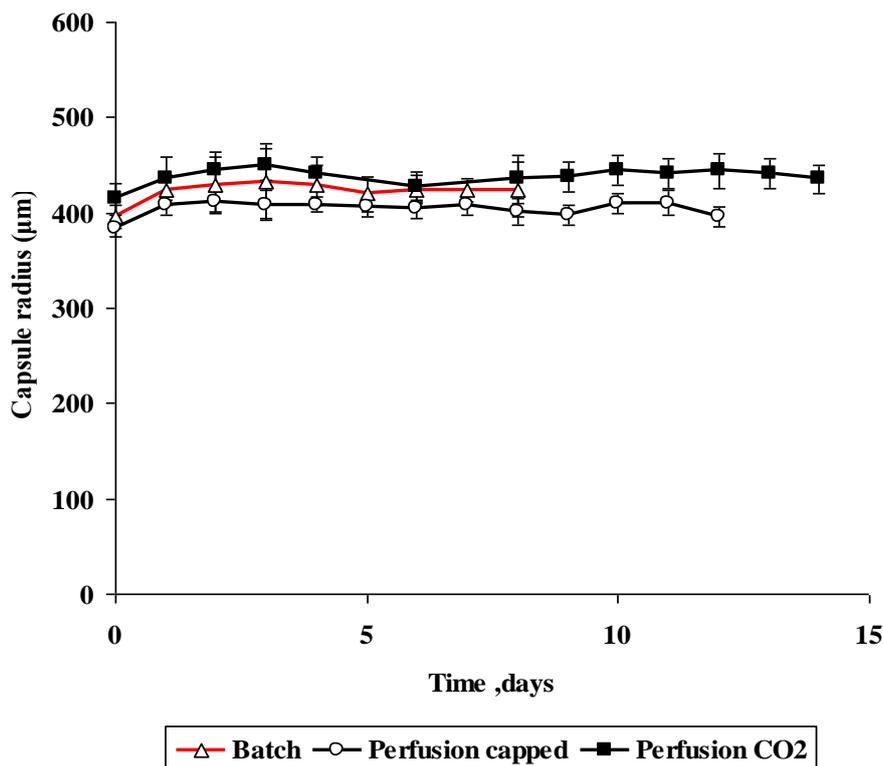


Figure 6.2.3.1.1.1: Average capsule radius size (μm) \pm STD over the duration of the cultivation period for triplicate encapsulated cultures performed under batch, perfusion capped (non-CO₂ incubator) and perfusion (CO₂ incubator). The size of approximately 30 capsules was measured at each time point and an average determined.

6.2.3.1.2: Capsule strength

The mechanical strength of the microcapsules in the batch and control-fed perfusion cultures was routinely evaluated in terms of Force (g)/capsule which the capsule could withstand using the Texture analyser.

Evaluation of Figure 6.2.3.1.2.1 below indicates a decrease in the force (g)/capsules over cultivation time, representative of a decrease in the mechanical resistance of the APA microcapsules throughout the culture period. The main decreases in mechanical resistance in all culture conditions were noted to occur from days 0 to 3, as has been previously noted in the other encapsulation experiments. Similarly to the batch microcapsules, the control-fed perfusion microcapsules did not reach the low level of mechanical resistance, around 0.07 g/capsules, until after day 3 (day 6 for CO₂ and day 7 for non-CO₂). After this time point, the mechanical resistance levels off. Nevertheless, the capsule in this study were still of a similar mechanical resistance to the minifors reactor batch and control-fed perfusion controls when high levels of capsule break-up were reported in Section 6.2.1.1.2. Due to the optimal shear environment associated with a shake flask culture, the fact that the capsules were weakened did not result in the same losses in capsule integrity that were noted in reactor control-fed perfusion cultures. Even with the introduction of perfusion on day 5, which brings about a medium equilibrium challenge to the already weakened capsules, no major capsule breakage was noted to occur. The method used to determine the force (g)/capsule analysed a layer of capsules at a time.

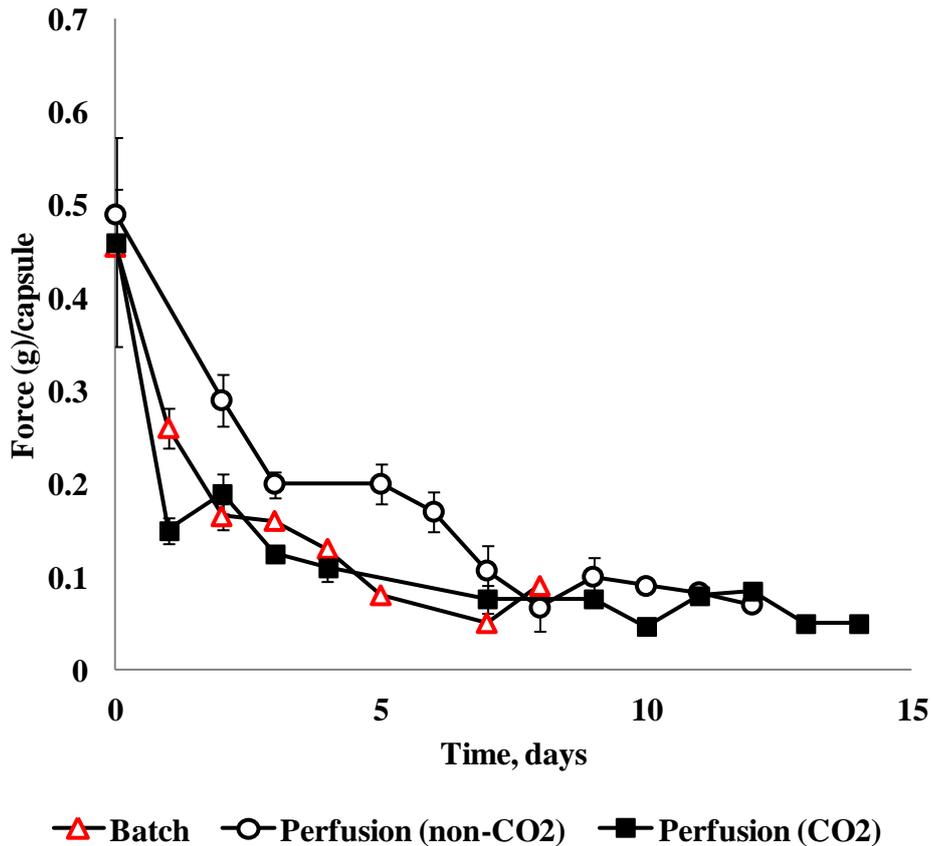


Figure 6.2.3.1.2.1: Mechanical resistance (force (g)/capsule) over cultivation period for 300 μm APA microcapsules containing CHO DP-12 cells cultivated under batch and control-fed perfusion conditions in 1 L Erlenmeyer shake flasks. A total of 3 layers of microcapsules were analysed on a daily basis for each of the cultures carried out. Values presented are average of triplicate analysis, \pm STD, for one of the encapsulated cultures carried out in each of the studies.

6.2.3.1.3: Capsule permeability

The permeability of the microcapsules to dextrans ranging in molecular weights from 10-500 kDa was determined for the 300 μm radius APA microcapsules. 300 μl of capsule suspension was incubated in 600 μl of a 0.05 % fluorescent dextran solution of a particular molecular weight. The capsules were incubated under agitation for 2 hours and analysed using confocal microscopy. Comparison of the initial concentration of dextran (C_0) in the capsule/dextran suspension and the final concentration of dextran (C_f) enabled determination of the permeability of microcapsules to varying molecular weights. The concentrations were determined by spectroscopy at 450 nm. As noted in Materials and

Methods section 2.3.7: C_0/C_f values equal to 1 indicate a lack of diffusion of the dextran into the microcapsule. C_0/C_f values of 1.5 are indicative of free diffusion. Values in the range of 1-1.5 indicate that diffusion of the dextran did occur, however a difficulty in equilibrium was noted.

Figure 6.2.3.1.3.1, 6.2.3.1.3.2, 6.2.3.1.3.3 below represent the C_0/C_f data obtained during the batch, perfusion non-CO₂ and perfusion CO₂ culture periods respectively. The data in each case did not determine the exact molecular weight cut-off point of the microcapsules. The aim of the analysis was to verify the permeability of the capsules to the rIgG1 protein (150 kDa) produced by the cells. In all cases the 150 kDa dextran approached a C_0/C_f value of 1.5, indicating free diffusion of species in this size range through the capsule membrane. As the mechanical resistance of the capsules were decreasing over time, it would be expected that the permeability of the microcapsules to dextrans would increase with increasing molecular weight over time. This was noted under all 3 culture conditions for the 250 kDa dextran. The 500 kDa dextran did not reach a point of ease of diffusion (i.e. a C_0/C_f approaching 1.5) at any stage in any of the culture conditions.

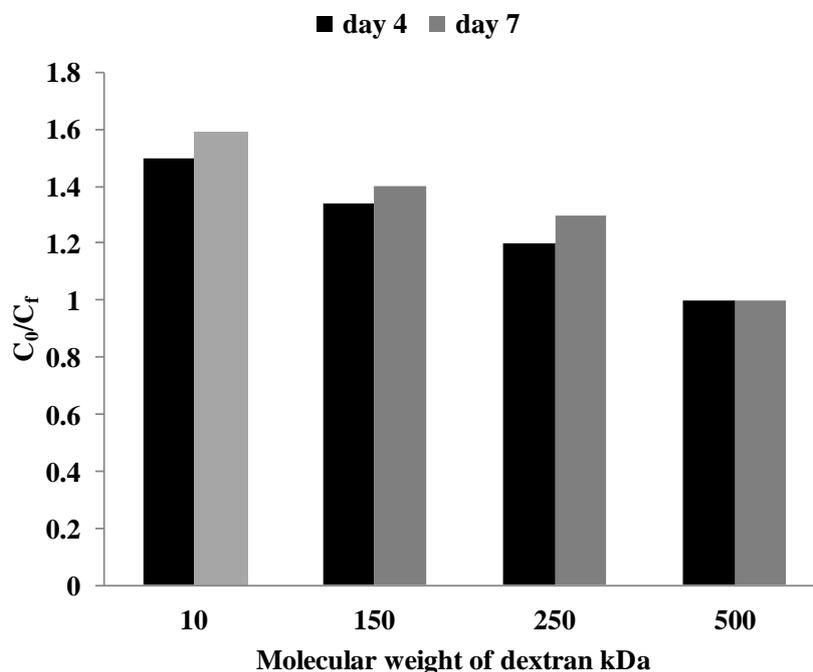


Figure 6.2.3.1.3.1: Permeability of 300 μm APA microcapsules to dextrans of varying molecular weight during a batch culture in 1 L Erlenmeyer shake flasks in a non-CO₂ incubator. Permeability is determined by the ratio of the initial concentration of dextran in a

microcapsule suspension to the concentration observed after a 2 hour incubation period with agitation. A ratio of 1.5 indicates free diffusion. A ratio of 1 is indicative of no permeability to the dextran by the capsules. The concentrations of dextran were determined spectroscopically at 450 nm.

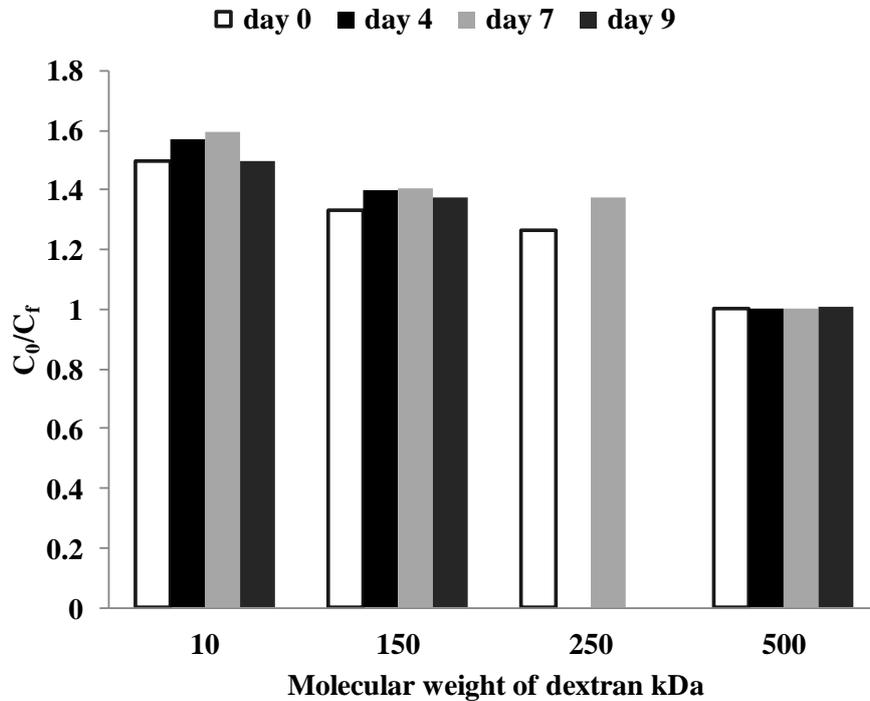


Figure 6.2.3.1.3.2: Permeability of 300 μm APA microcapsules to dextrans of varying molecular weight during a control-fed perfusion culture in 1 L Erlenmeyer shake flasks in a non- CO_2 incubator. Permeability is determined by the ratio of the initial concentration of dextran in a microcapsule suspension to the concentration observed after a 2 hour incubation period with agitation. A ratio of 1.5 indicates free diffusion. A ratio of 1 is indicative of no permeability to the dextran by the capsules. The concentrations of dextran were determined spectroscopically at 450 nm

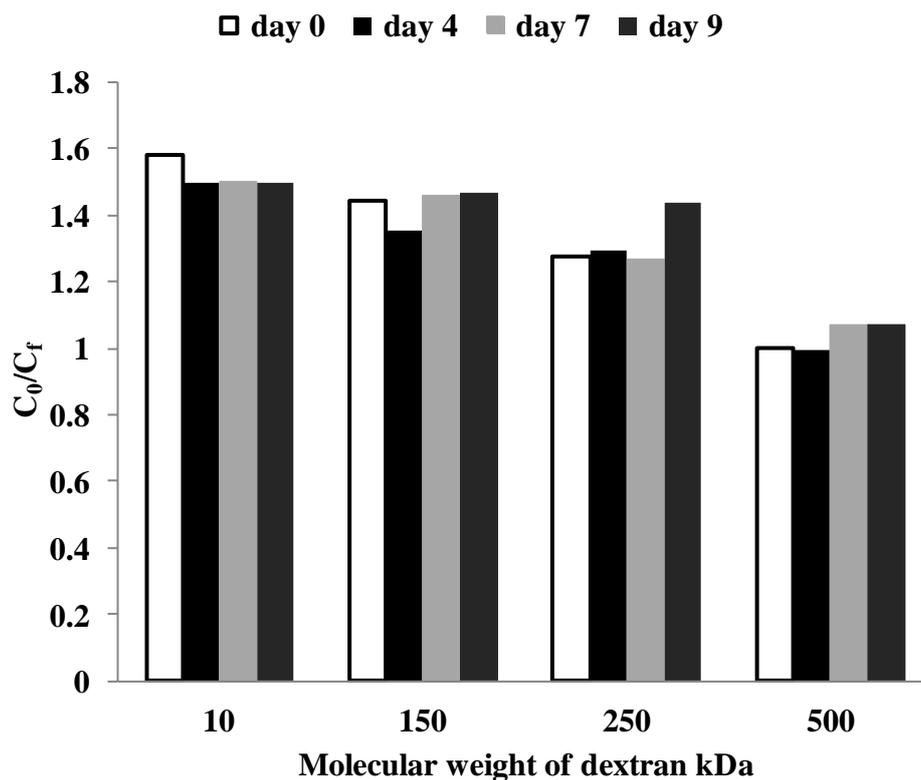


Figure 6.2.3.1.3.3: Permeability of 300 μm APA microcapsules to dextrans of varying molecular weight during a control-fed perfusion culture in 1 L Erlenmeyer shake flasks in a 5 % CO_2 incubator. Permeability is determined by the ratio of the initial concentration of dextran in a microcapsule suspension to the concentration observed after a 2 hour incubation period with agitation. A ratio of 1.5 indicates free diffusion. A ratio of 1 is indicative of no permeability to the dextran by the capsules. The concentrations of dextran were determined spectroscopically at 450 nm

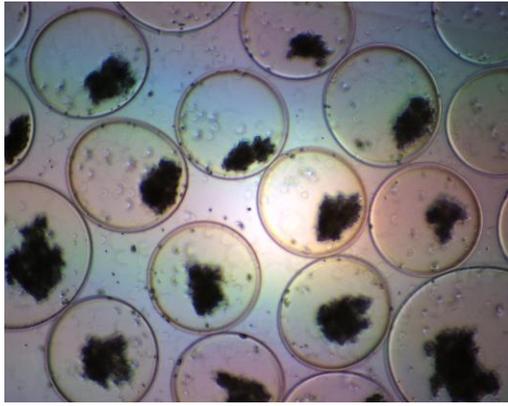
6.2.3.2: Growth of encapsulated cells in 1L Erlenmeyer shake flasks under conditions of batch (non-CO₂ incubator), perfusion (non-CO₂ incubator) and perfusion (CO₂ incubator) mode of cultivation.

A controlled-fed perfusion strategy was applied to a CHO DP-12 cell line encapsulated in APA microcapsules and cultured in 1 L Erlenmeyer shake flasks in both unvented (non-CO₂ incubator) and vented (5 % CO₂ incubator) shake flasks. Cells were seeded at average seeding densities 0.36×10^6 cells/ml_{reactor} and incubated at 37 °C and 100 rpm agitation. Figures 6.2.3.2.1 below allows direct visualisation and comparison of the growth of cells inside the capsules for the controlled-fed perfusion cultures in comparison to the batch culture previously carried out in 1 L Erlenmeyer shake flasks in Section 6.2.2. The maximum viable cell density ceased to increase beyond day 6 in batch cultures resulting in a total of 5 % capsule colonisation. Cell numbers continued to increase until day 8 in the controlled-fed perfusion culture carried out under non-CO₂ conditions. Capsule colonisation in the non-CO₂ incubated flasks was determined to be ~11 %. Further colonisation of the microcapsules occurred in cultures incubated in the 5 % CO₂ incubator, with the degree of colonisation being 27 % when maximum cell density was reached on day 9. These results are verified by the data presented in Figure 6.2.3.2.2 which illustrates the concentration of cells in each culture (cells/ml_{capsules}) over the cultivation period. The max viable cell density (cells/ml_{capsules}) obtained in the perfusion CO₂ culture was ~5- fold higher than that achieved in the batch culture and 2.5- fold higher than that achieved in the controlled-fed perfusion (non-CO₂) culture.

Figure 6.2.3.2.3 below illustrates the LN plot of the viable cell density (cells/ml_{reactor}) over the cultivation period, for the batch and controlled-fed perfusion cultures (non-CO₂ and CO₂). Both controlled-fed perfusion cultures acted as batch cultures until day 5 essentially. The growth rates of the 3 cultures from days 1-5, presented in Table 6.2.3.2.1 are not significantly different from each other, as expected. Although there was no significant difference in the growth rate of the cells in the cultures from days 1-5, it is important to note that there was a lower viable cell number (cells/ml_{reactor}), Figure 6.2.3.2.4, present in the controlled-fed perfusion culture carried out in the 5 % CO₂ incubator. This is the culture in which had the lowest mean cell specific growth rate from days 1-5. The significance of this lower viable cell number may be important in discussing possible differences in volumetric productivities during days 1-5 in the cultures. As noted in Figure 6.2.3.2.3, the batch culture ceased exponential growth on day 5 with one more increase in viable cell number occurring on day 6

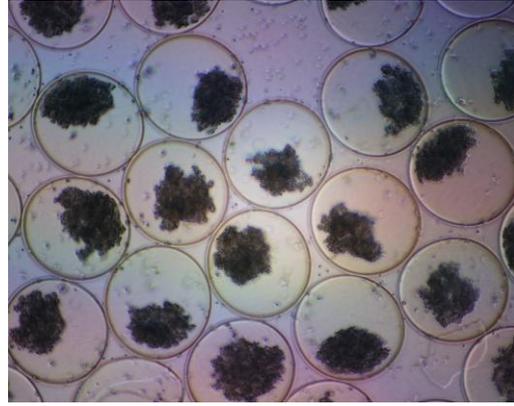
before the cells entered a stationary phase until day 8. For the controlled-fed perfusion (non-CO₂) culture, cell number continued to increase from days 5-8. However, the calculated growth rate (0.346 day⁻¹) during this time was significantly lower (~ 50 %) than that observed during the exponential growth period (0.688 day⁻¹). This culture entered a stationary phase until day 11 (as determined by LN plots) before a subsequent fall off in cell viability was observed, Figure 6.2.3.2.5. As noted in Table 6.3.3.2.1, the continued cell growth beyond day 5 resulted in an increased viable cell density (cells/ml_{reactor}) ~ 2 fold higher than that obtained in the batch culture. The controlled-fed perfusion culture conducted in the CO₂ incubator, maintained the maximum exponential growth rate from days 5-9 when perfusion was initiated on days 5. After day 9, no further increases in maximum cell number were achieved and the cells entered a stationary phase until a fall off in cell viability was noted on day 13, Figure 6.2.3.2.5. As expected, the data presented in Table 6.2.3.2.1 illustrates an ~ 5 and ~2.5 fold increases in maximum cell number achieved in comparison to the batch and controlled-fed perfusion (non-CO₂) cultures, respectively.

Comparison of Figure 6.2.3.2.2 and Figure 6.2.3.2.4 gives an indication of the suitability of the microcapsules in these conditions as retention devices for perfusion systems. Increases in cells per ml_{capsule} detailed in Figure 6.2.3.2.2 have directly been translated into increases in cells per ml_{vessel} in Figure 6.2.3.2.4



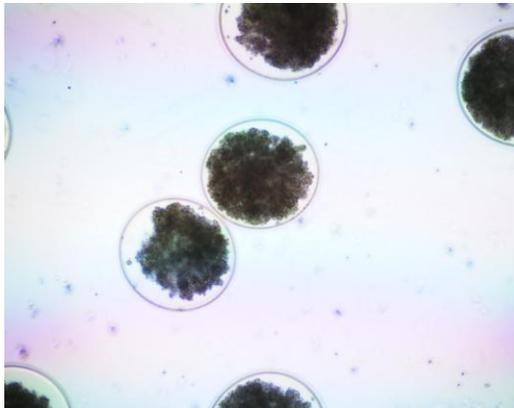
40x

Batch shake flask day 6

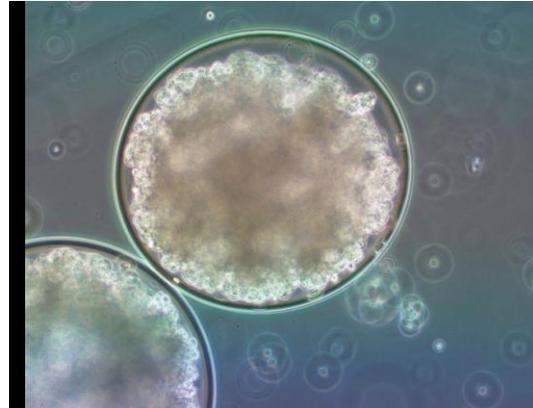


40x

Perfusion (non-CO₂) incubator day 9



40x



100x

Perfusion (CO₂ incubator) day 9

Figure 6.2.3.2.1: Microscopic analysis (unstained) of microcapsule colonisation by cells cultured under batch and controlled-fed perfusion modes of cultivation in both non-CO₂ and CO₂ incubators. Cells were originally seeded at 0.5×10^6 cells/ml alginate and which was extruded through a 300 μ m nozzle as part of the liquid core microcapsule formation. The resulting microcapsules were used to seed 1 L Erlenmeyer shake flask cultures with a ratio of 100 ml capsules and 300 ml media.

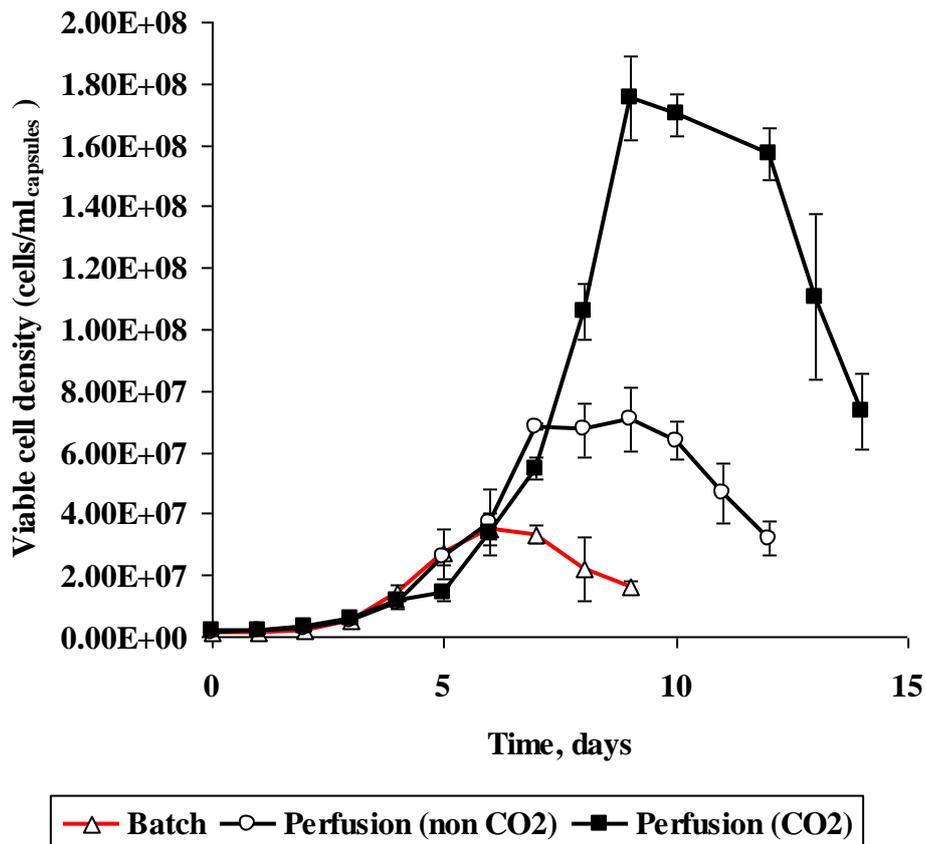


Figure 6.2.3.2.2: Growth of CHO cells cultures in 300 μm liquid core APA microcapsules under batch and controlled-fed perfusion (non- CO_2 and CO_2 incubators) cultivation conditions in 1 L Erlenmeyer shake flask. Cell numbers were enumerated on a daily basis using trypan blue exclusion method and a Neubauer haemocytometer. Encapsulated cells were liberated prior to counting via extrusion of a capsule/PBS suspension through a 300 μm needle 3 times. Duplicate cell counts were performed. The cell concentration is given with respect to the capsule's volume. Values are the average (\pm STD) of triplicate cultures performed in parallel for each condition tested.

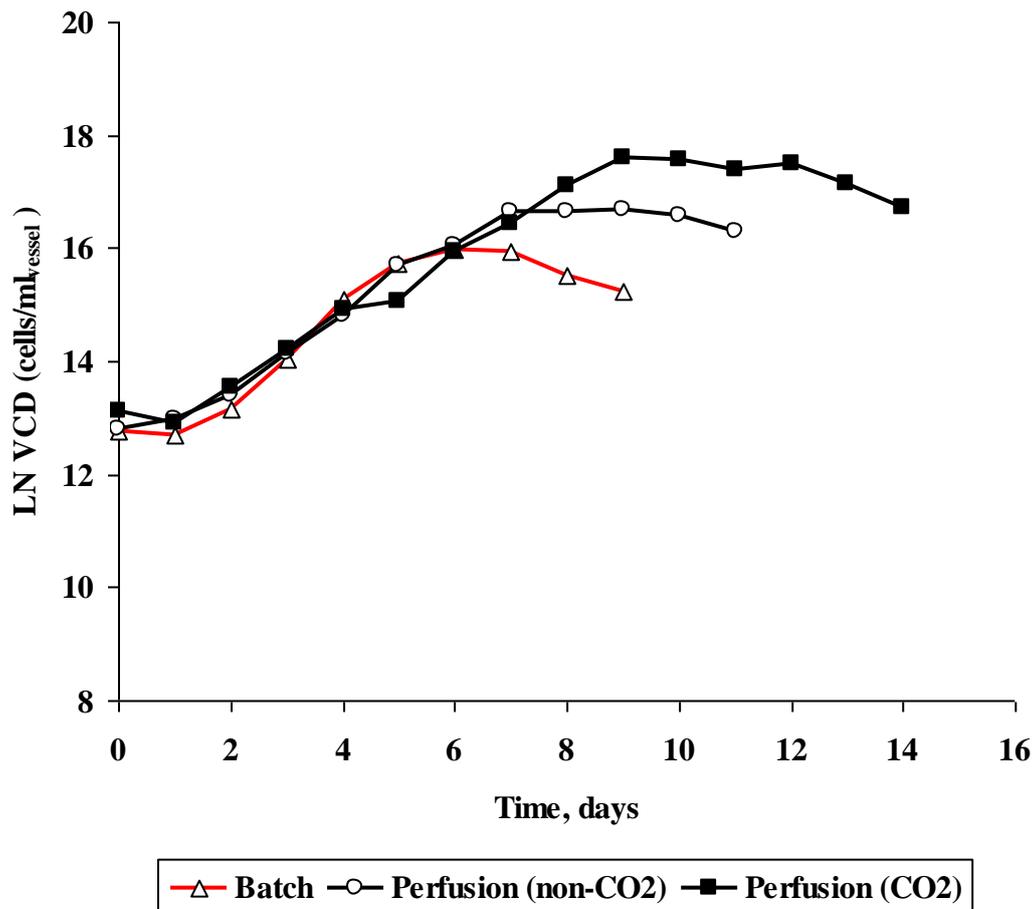


Figure 6.2.3.2.3: LN plot of viable cell densities (cells/ml_{vessel}) over the cultivation period for cells encapsulated in 300 μm APA microcapsules and cultured under batch and controlled-fed perfusion (non-CO₂ and CO₂ incubator) modes of cultivation in 1 L Erlenmeyer shake flasks. Cell numbers were enumerated on a daily basis using trypan blue exclusion method and a Neubauer haemocytometer. Encapsulated cells were liberated prior to counting via extrusion of a capsule/PBS suspension through a 300 μm needle 3 times. Duplicate cell counts were performed and the average recorded.

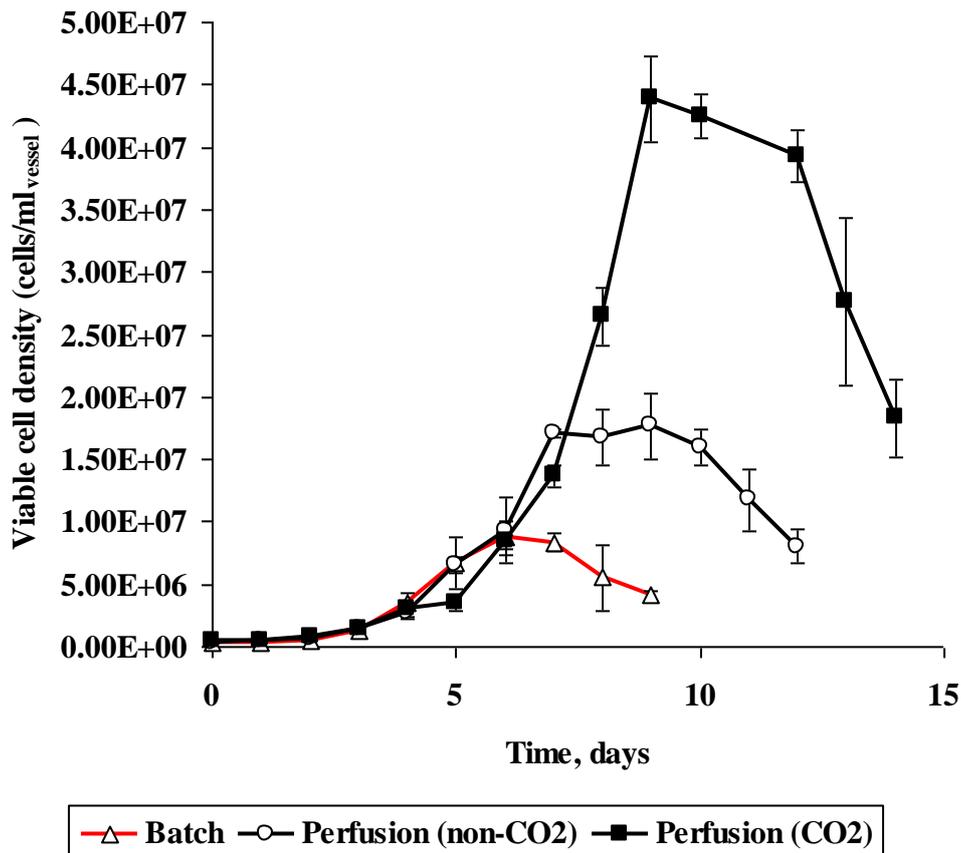


Figure 6.2.3.2.4: Growth of CHO cells cultures in 300 μm liquid core APA microcapsules under batch and controlled-fed perfusion (non-CO₂ and CO₂ incubators) cultivation conditions in 1 L Erlenmeyer shake flask. The cell concentration is given with respect to the working volume in the shake flask. Cell numbers were enumerated on a daily basis using trypan blue exclusion method and a Neubauer haemocytometer. Encapsulated cells were liberated prior to counting via extrusion of a capsule/PBS suspension through a 300 μm needle 3 times. Duplicate cell counts were performed and the average recorded. Values are the average (+/- STD) of triplicate cultures performed in parallel for each condition tested.

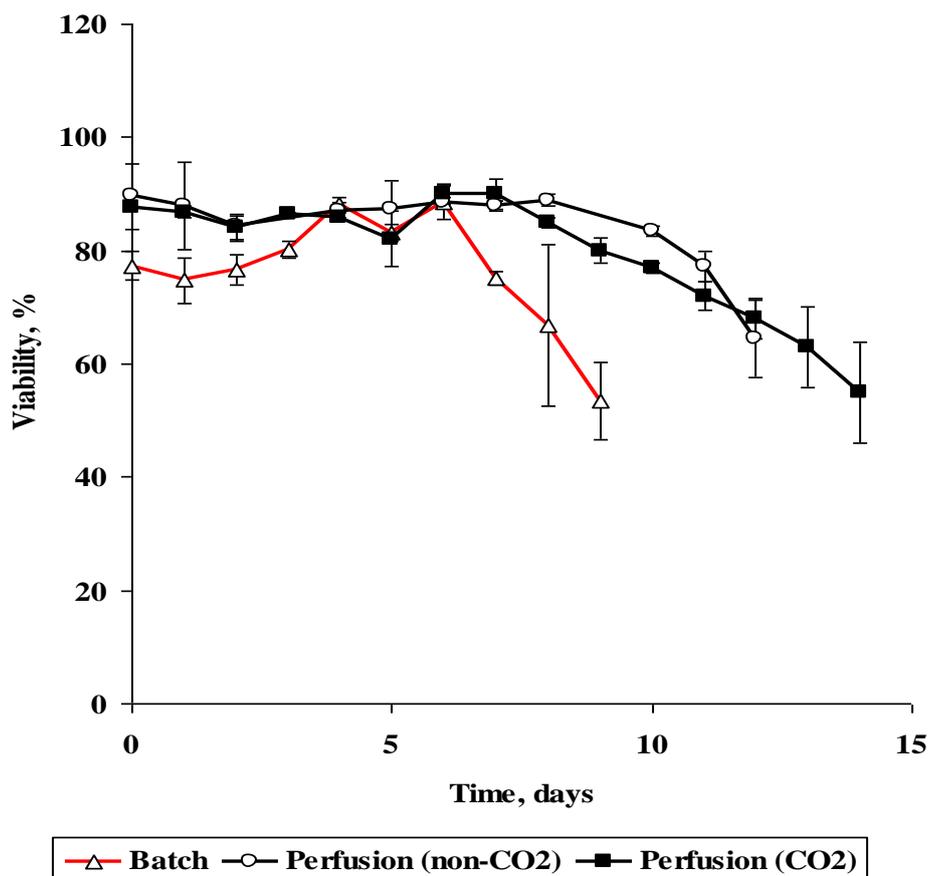


Figure 6.2.3.2.5: % Viability of CHO cells encapsulated in 300 μm liquid capsules and cultured in 1 L Erlenmeyer shake flask under batch and controlled-fed perfusion (non-CO₂ and CO₂ incubator) modes of cultivation. % viability was determined by the enumeration of total and live cell numbers present in the culture using trypan blue exclusion method and Neubauer haemocytometer. % viability is expressed as a percentage of live cells over dead cells present. Cell counts were performed in duplicate and average % viability determined on a daily basis. Values are average (+/- STD) of triplicate cultures performed in parallel for the culture conditions tested.

Table 6.2.3.2.1: Calculated maximum specific growth rates displayed by cells encapsulated in 300 μm liquid capsules and cultures in 1L Erlenmeyer shake flasks under batch and controlled-fed perfusion (non-CO₂ and CO₂ incubator) cultivation conditions. The growth rate was calculated from a plot of the logarithm of the viable cell density over the cultivation time (days). The maximum viable cell density is presented with respect to per ml_{vessel}. Values are average (+/- STD) of those determined for triplicate cultures performed in parallel for the culture conditions tested.

Culture	μ_{max} (day ⁻¹) days 1-5	μ_{max} (day ⁻¹) day 5-stationary phase	Max VCD (10 ⁶ cells/ml _{vessel})
Batch	0.789 +/- 0.060	NA	9.21 +/- 1.15
Perfusion (non-CO ₂)	0.688 +/- 0.086	0.346 +/- 0.44	18.5 +/- 1.33
Perfusion (CO ₂)	0.557 +/- 0.071	0.622 +/- 0.053	44.9 +/- 2.54

6.2.3.3: Metabolic analysis of encapsulated cells cultured in 1L Erlenmeyer shake flasks under conditions of batch (non-CO₂ incubator), controlled-fed perfusion (non-CO₂ incubator and CO₂ incubator) modes of cultivation.

All shake flask cultures were seeded in 300 ml of ExCell CHO 325 PF media supplemented with 4 mM L-glutamine. The concentration of glucose in the commercially available media is expected to be 17.4 mM. The concentration of the major carbon sources, glutamine and glucose, and the by-product, ammonia were measured from media samples taken from all cultures on a daily basis using commercially available enzymatic assay kits. The controlled-fed perfusion cultures essentially acted as batch cultures until day 5, the point at which it was estimated that the concentration of glucose and glutamine were insufficient to meet the needs of the predicted cell numbers present on the following day. Such predictions were made based on the cellular growth and cell specific consumption rates. Perfusion was initiated on day 5 by the removal of 100 ml of spent media from the culture flasks and replacement with 100 ml of fresh media of a daily basis. The concentration of the metabolites glucose and glutamine in the fresh media were adjusted on a daily basis to meet the requirements of the actively growing cells. The perfusion rate remained constant for the duration of the controlled-fed perfusion culture in the non-CO₂ incubator. The rate was adjusted to 2 media changes per day for the controlled-fed perfusion culture in the 5 % CO₂ incubator after day 9. This was due to the fact that the concentration of ammonia in the culture was reaching levels which are normally higher than that noted in batch cultures < 5 mM. Also, studies conducted in Chapter 3 of the thesis did indicate alterations in the

glycosylation profile in regards to the level of sialylation when initial ammonia concentrations in the culture were in the range of 5-15 mM.

Due to the fact that the control-fed perfusion cultures acted as batch cultures until day 5, there should be no significant differences in the metabolic profiles displayed by cells cultured in the batch and controlled-fed perfusion (non-CO₂ incubator) cultures during this time point. Any alterations noted in metabolic activity for cells cultured in the controlled-fed perfusion cultures in the 5 % CO₂ incubator during this time point would be due to the influence of the 5 % CO₂ atmosphere. Table 6.2.3.3.1 and Table 6.2.3.3.3 indicate the volumetric and cell specific consumption and production rates displayed by the cells for glucose, glutamine, lactate and ammonia. Lactate concentrations throughout the culture period were determined using HPLC after culture termination from offline samples stored at -80 °C. There was no significant difference in the volumetric or cell specific consumption or production rates of the metabolites glucose, glutamine and lactate during days 1-5 of the culture periods for all culture conditions tested. There was a significantly lower volumetric ammonia production rate during this time period in the controlled-fed perfusion culture conducted in the 5 % CO₂ incubator. This is however as a direct result of the lower cell number present in this culture on day 5, which was noted above in Section 6.3.3.2. The cell specific ammonia production rate was not affected by the cultivation of the cells in the 5 % CO₂ incubator. This data is verified by the plots of glucose, lactate, glutamine and ammonia in which the trends for consumption and production from days 1-5 are noted to be similar, (Figure 6.2.3.3.1-6.2.3.3.4)

In the batch culture all of the glucose and glutamine were depleted by days 9 and 6 respectively. Lactate and ammonia production resulted in the accumulation of a maximum concentration of ~17.6 and ~3.7 mM respectively. The yield of lactate on glucose and ammonia on glutamine are therefore as expected, 1.01 +/- 0.05 and 1.22 +/- 0.11 respectively, Table 6.2.3.3.4. In the control-fed perfusion non-CO₂ culture, the glucose concentration in the culture was increased from ~ 6 mM to ~ 20 mM on day 5, due to the prediction that the cells would continue to grow at the same exponential growth rate from days 1-5. However due to a fall off in the growth rate over the period of increasing cell number (days 5-8), an over estimation was made in the concentration of glucose required by the cells over this time period. There was also a 32 % decrease in the specific glucose consumption rate displayed by the cells over this time period in comparison to that observed during the exponential growth period (days 1-5), Table 6.2.3.3.3. For this reason, no additional glucose, bar the

concentration contained in the commercial media, was added to the cultures over the extended growth period. For the metabolite glutamine, there was no significant difference in the specific consumption rate displayed by the cells over the extended growth period during days 5-8 in comparison to that observed during days 1-5, Tables 6.2.3.3.2 & 6.2.3.3.3. The concentration of glutamine during the time period of days 5-8 varied in the range of ~1-3 mM during this time period. It may therefore be stated that the controlled-fed perfusion culture did perform well in that the concentration of metabolites were maintained in excess quantities to meet the requirements of the predicted cell numbers for the following days. Neither glucose nor glutamine were limiting at the end of the stationary growth period.

The specific lactate production rate was similar to the specific glucose consumption rate over the exponential growth period (days 1-5) in the control-fed perfusion non-CO₂ culture. Although the specific glucose consumption rate declined over the extended growth period in comparison to the exponential growth period, the specific lactate production rate remained unchanged, suggesting a movement to less efficient glucose metabolism in this culture. The maximum lactate concentration reached in this culture was ~ 22 mM. The overall yield of lactate on glucose over the entire culture period was 1.25 +/- 0.07 mmoles/mmoles, which is 24 % higher than that observed in the batch cultures, due to the shift to less efficient glucose metabolism, Table 6.2.3.3.4. There was a 40 % reduction in the specific ammonia production rate over the extended growth period (days 5-8) in comparison to that which was observed in the exponential growth period, Table 6.2.3.3.3 This decreased ammonia production rate indicated more favourable glutamine metabolism over the extended growth period. The maximum ammonia concentration at any point in this culture was ~2.5 mM. The yield of ammonia on glutamine over the entire culture period was 0.88 +/- 0.15 mmoles/mmoles, which is 28 % lower than that observed in the batch culture, which can be attributed to the more efficient glutamine metabolism observed over the extended growth period, Table 6.2.3.3.4

For the case of the control-fed perfusion culture conducted in the 5 % CO₂ incubator, the application of the control-fed perfusion strategy to the culture from day 5 onwards, resulted in an increased exponential growth until day 9, as noted in Section 6.2.3.2 above. The concentration of glucose added to this culture in the perfusion media on day 5 was determined based on the cell specific glucose consumption rate over the exponential period. The cell number present on the following day was as predicted due to persistence of the exponential growth rate beyond day 5. However there was a decrease in the cell specific

consumption rate for glucose during this time period, by approximately 50 %, which resulted in an over estimation of the concentration of glucose in the perfusion media, Table 6.2.3.3.1 & 6.2.3.3.3. As noted from the plot of glucose over the culture period, Figure 6.2.3.3.1, approximately only 50 % of the glucose added was consumed between days 5 and 6. This corresponds with the 50 % decrease in the cell specific glucose consumption rate over the extended growth period (days 5-9). The control-fed perfusion strategy did work well for this culture as the forward day predictions for cell number and glucose concentration resulted in a maintained glucose concentration range of between 7-12 mM. For the metabolite glutamine, the cell specific consumption rate also decreased, in this case by 60 %, over the extended growth period (days 5-9) in comparison to that calculated for the exponential phase (days 1-5), Table 6.2.3.3.2 & 6.2.3.3.3. No changes were observed in the specific glutamine consumption rate for the same culture conditions, however under a non-CO₂ environment. The control-fed perfusion strategy did also work well for this control in regards to maintaining a glutamine concentration in the range of 1-7 mM throughout the culture period. It may be noted that for the control-fed perfusion culture performed in the 5 % CO₂ incubator, a greater decrease in the glucose consumption rate was observed over the extended growth period, in comparison to that observed over the same period for the control-fed perfusion non-CO₂ incubated culture.

A 60 % reduction in the specific lactate production rate was observed over the extended growth period (days 5-9) in comparison to that observed in the exponential growth period for the control-fed perfusion culture incubated in the 5 % CO₂ incubator, Table 6.2.3.3.1 & 6.2.3.3.3. There was a similar decrease in both the glucose and the lactate cell specific consumption and production rates (+/- 5 %), the cells cultured in these conditions. The cells did therefore not experience the same shift to inefficient glucose metabolism over the extended growth period as was observed for the control-fed perfusion cultures conducted in the non-CO₂ incubator. The maximum lactate concentration obtained in this culture was ~19.15 mM. The yield of lactate on glucose over the entire culture period (0.93 +/- 0.06 mmoles/mmoles) was 26 % lower than that observed in the non-CO₂ incubated control-fed perfusion culture. A 3.6- fold decrease in the specific ammonia production rate was observed over the extended growth period in comparison to that observed during the exponential growth period for cells cultures in the control-fed perfusion cultures in the 5 % CO₂ incubator, Table 6.2.3.3.2 & 6.2.3.3.3. This reduction was significantly higher than the reduction observed in glutamine between the exponential growth period and the extended

growth period, indicating an increased glutamine metabolic efficiency. The shift to efficient glutamine metabolism was even more profound in this culture, in comparison to the same culture conducted in the non-CO₂ incubator due to the lower yield observed (-30 %) in the control-fed perfusion culture in the 5 % CO₂ incubator in comparison to that observed for the culture in the non-CO₂ incubator. Although a lower yield of ammonia and a lower specific production rate of ammonia was observed in the 5 % CO₂ culture, in comparison to the non-CO₂ culture, due to the increased number of cells present, the concentration of ammonia present in the culture on day 9 did approach ~5 mM, actual value (4.4 mM), the perfusion rate was subsequently increased to two medium changes per day which maintained maximum ammonia concentration observed in this culture at ~ 5 mM.

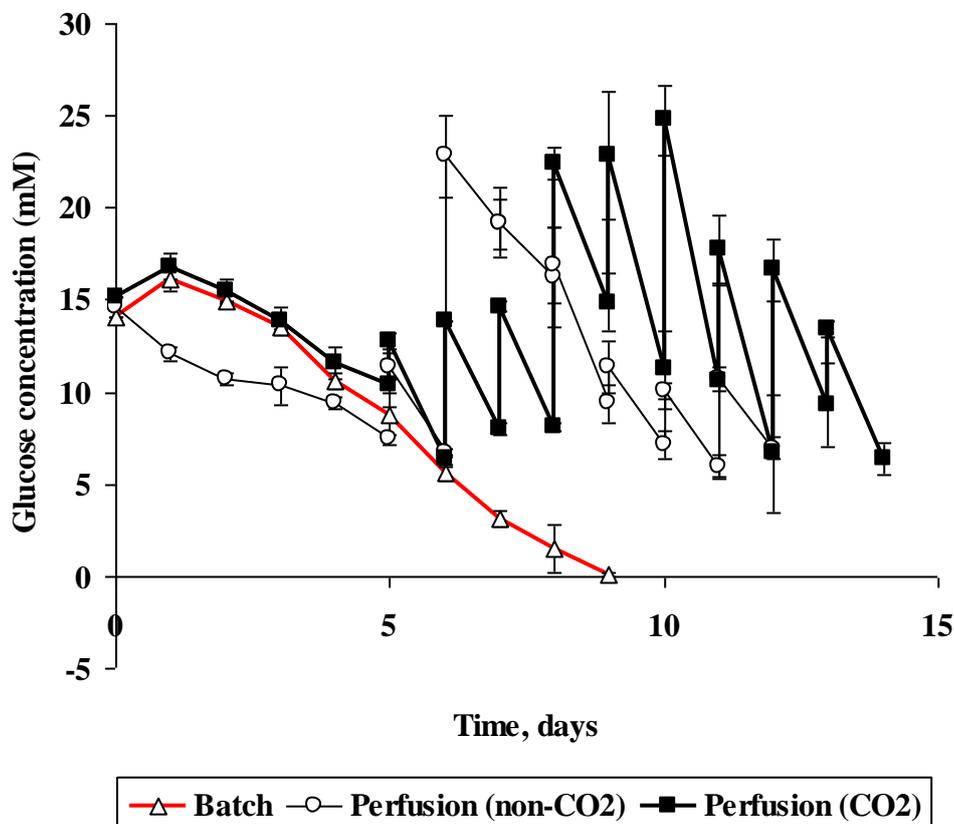


Figure 6.2.3.3.1: Concentration of glucose present in encapsulated batch and controlled-fed perfusion cultures (non-CO₂ and CO₂), performed in 1 L Erlenmeyer shake flasks, over the duration of the respective cultivation period. The concentrations were determined offline using a commercial available enzymatic assay kit. Values are average (+/- STD) of triplicate cultures performed in parallel for the culture conditions tested.

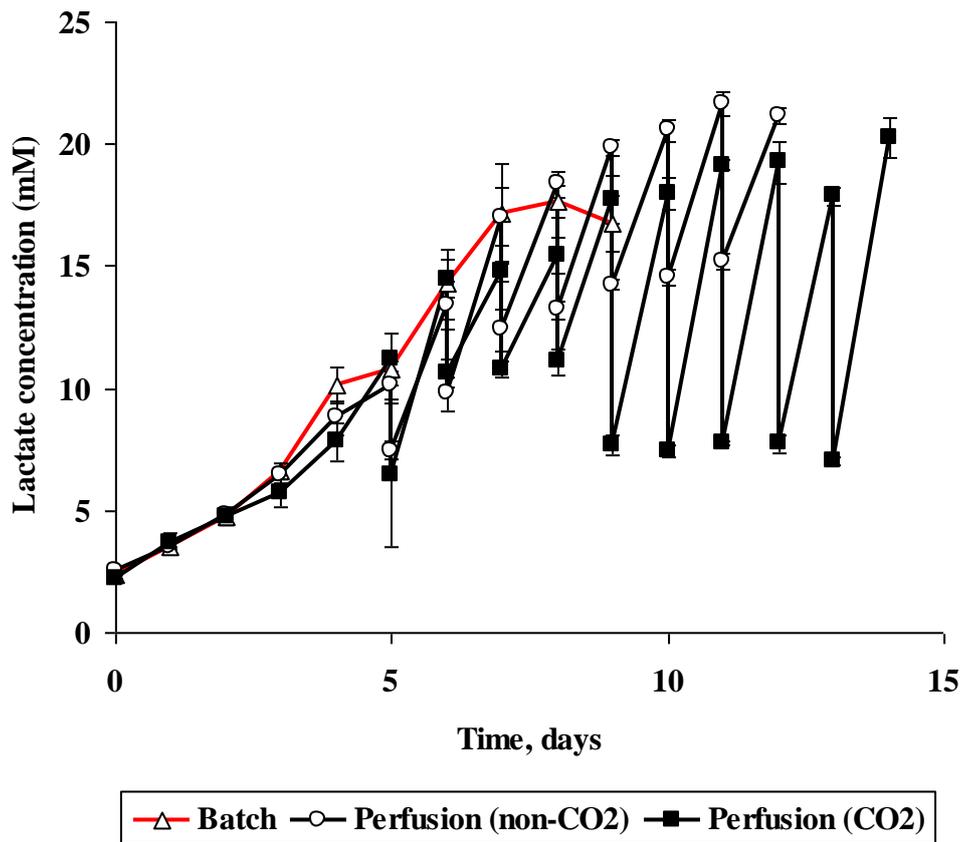


Figure 6.2.3.3.2: Concentration of lactate present in batch and controlled-fed perfusion (non-CO₂ and CO₂) cultures, performed in 1 L Erlenmeyer shake flasks, over the duration of the respective cultivation period. The concentrations were determined offline HPLC analysis using Supelco H column. Values are average (+/- STD) of triplicate cultures performed in parallel for the culture conditions tested.

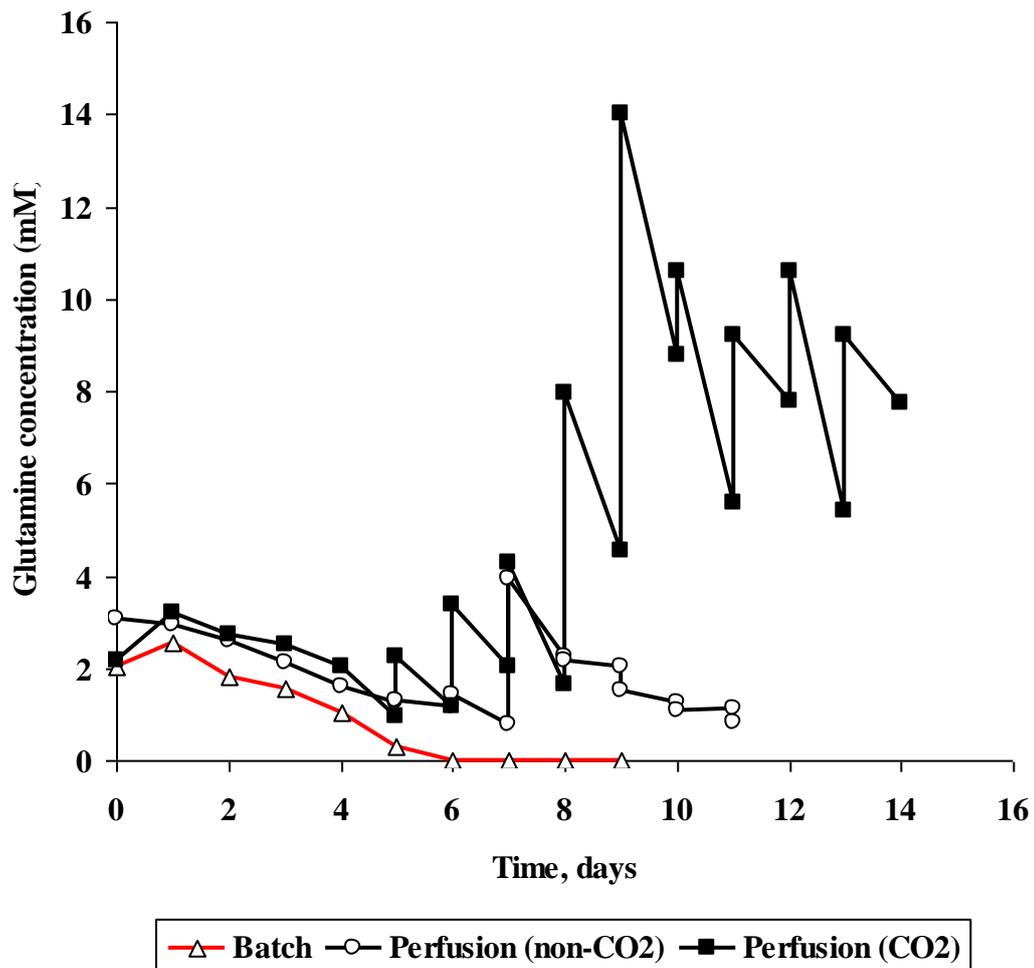


Figure 6.2.3.3.3: Concentration of glutamine present in batch and controlled-fed perfusion cultures (non-CO₂ and CO₂), performed in 1 L Erlenmeyer shake flasks, over the duration of the respective cultivation period. The concentrations were determined offline using a commercial available enzymatic assay kit. Values are average (+/- STD) of triplicate cultures performed in parallel for the culture conditions tested.

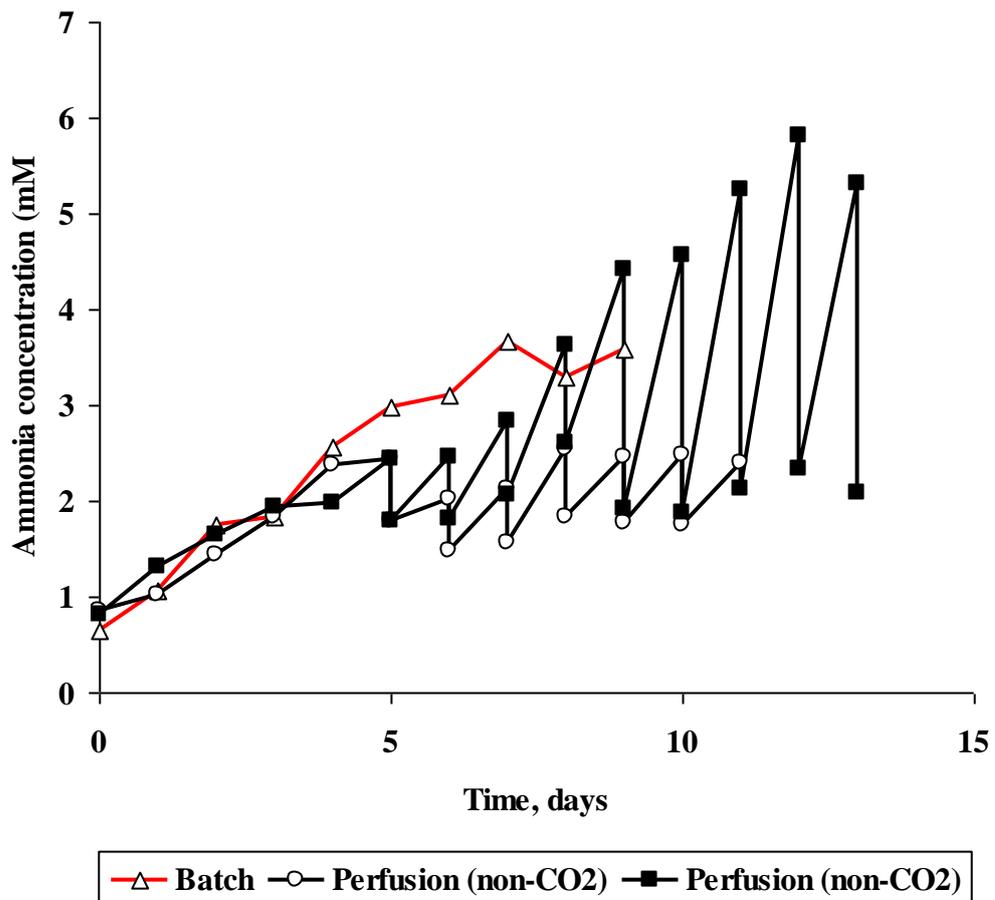


Figure 6.2.3.3.3: Concentration of ammonia present in batch and controlled-fed perfusion cultures (non-CO₂ and CO₂), performed in 1 L Erlenmeyer shake flasks, over the duration of the respective cultivation period. The concentrations were determined offline using a commercial available enzymatic assay kit. Values are average (+/- STD) of triplicate cultures performed in parallel for the culture conditions tested.

Table 6.2.3.3.1: All glucose and lactate volumetric (mmoles/day) and specific (mmoles/10⁶ cells/day) consumption and production kinetics calculated over the exponential phase (days 1-5), before control-fed perfusion strategy was applied to the non-CO₂ and CO₂ cultures. Values are average (+/- STD) of those calculated for triplicate cultures performed in parallel for the culture conditions tested.

Culture	Glucose consumption rate (mM/day) day 1-5	Lactate production rate (mM/day) day 1-5	q_{glucose} (mmoles/10⁶ cells/day) day 1-5	q_{lactate} (mmoles/10⁶ cells/day) day 1-5
Batch	1.89 +/- 0.06	1.99 +/- 0.18	$2.69 * 10^{-4}$ +/- $7.87 * 10^{-5}$	$3.42 * 10^{-4}$ +/- $8.24 * 10^{-5}$
Perfusion (non-CO ₂)	1.83 +/- 0.04	1.87 +/- 0.04	$2.14 * 10^{-4}$ +/- $1.83 * 10^{-5}$	$2.05 * 10^{-4}$ +/- $3.37 * 10^{-5}$
Perfusion (CO ₂)	1.89 +/- 0.07	1.9 +/- 0.04	$2.1 * 10^{-4}$ +/- $1.57 * 10^{-5}$	$2.16 * 10^{-4}$ +/- $3.79 * 10^{-6}$

Table 6.2.3.3.2: All glutamine and ammonia volumetric (mmoles/day) and specific (mmoles/10⁶ cells/day) consumption and production kinetics calculated over the exponential phase (days 1-5), before control-fed perfusion strategy was applied to the non-CO₂ and CO₂ cultures. Values are average (+/- STD) of those calculated for triplicate cultures performed in parallel for the culture conditions tested.

Culture	Glutamine consumption rate (mM/day) day 1-5	Ammonia production rate (mM/day) day 1-5	$q_{\text{glutamine}}$ (mmoles/10⁶ cells/day) day 1-5	q_{ammonia} (mmoles/10⁶ cells/day) day 1-5
Batch	0.53 +/- 0.05	0.46 +/- 0.01	$7.81 * 10^{-5}$ +/- $2.95 * 10^{-5}$	$5.98 * 10^{-5}$ +/- $1.98 * 10^{-5}$
Perfusion (Non-CO ₂)	0.44 +/- 0.02	0.44 +/- 0.02	$5.72 * 10^{-5}$ +/- $6.02 * 10^{-6}$	$4.28 * 10^{-5}$ +/- $6.08 * 10^{-6}$
Perfusion (CO ₂)	0.54 +/- 0.01	0.28 +/- 0.02	$1.02 * 10^{-4}$ +/- $3.09 * 10^{-5}$	$6.59 * 10^{-5}$ +/- $1.06 * 10^{-5}$

Table 6.2.3.3.3: All specific glucose, lactate, glutamine and ammonia specific consumption and production rates (mmoles/10⁶ cells/ml) over the extended growth period for control-fed perfusion non-CO₂ (days 5-8) and control-fed perfusion CO₂ (days 5-9). Values are average (+/- STD) of those calculated for triplicate cultures performed in parallel for the culture conditions tested.

Culture	q_{glucose} (mmoles/10⁶ cells/day)	q_{lactate} (mmoles/10⁶ cells/day)	$q_{\text{glutamine}}$ (mmoles/10⁶ cells/day)	q_{ammonia} (mmoles/10⁶ cells/day)
Perfusion (non-CO ₂)	1.45 * 10 ⁻⁴ +/- 2.27 * 10 ⁻⁵	2.01 * 10 ⁻⁴ +/- 4.77 * 10 ⁻⁵	3.93 * 10 ⁻⁵ +/- 1.18 * 10 ⁻⁵	2.57 * 10 ⁻⁵ +/- 4.16 * 10 ⁻⁶
Perfusion (CO ₂)	1.00 * 10 ⁻⁴ +/- 9.19 * 10 ⁻⁶	8.46 * 10 ⁻⁵ +/- 1.59 * 10 ⁻⁵	4.08 * 10 ⁻⁵ +/- 3.95 * 10 ⁻⁶	1.85 * 10 ⁻⁵ +/- 2.18 * 10 ⁻⁶

Table 6.2.3.3.4: All yields of by-product/metabolite for batch and control-fed perfusion (non-CO₂ and CO₂) cultures performed in 1 L Erlenmeyer shake flasks over the cultivation period. Values are average (+/- STD) of those calculated for triplicate cultures performed in parallel for the culture conditions tested.

Culture	$Y_{\text{lactate/glucose}}$ (mmol/mmol)	$Y_{\text{ammonia/glutamine}}$ (mmol/mmol)
Batch	1.01 +/- 0.05	1.22 +/- 0.11
Perfusion (non-CO ₂)	1.25 +/- 0.07	0.88 +/- 0.15
Perfusion (CO ₂)	0.93 +/- 0.06	0.61 +/- 0.06

6.2.3.4: Productivity analysis of encapsulated cells cultured in 1L Erlenmeyer shake flasks under conditions of batch (non-CO₂ incubator), controlled-fed perfusion (non-CO₂ incubator and CO₂ incubator) modes of cultivation.

To evaluate the effect of the controlled-fed perfusion mode of cultivation on rIgG1 production, media samples were removed from both the batch and controlled-fed perfusion cultures on alternating days (5, 7, 9, 11, 13, 14). As explained in Section 2.3.6., of the Materials and Methods Section, rIgG1 was purified from media samples removed from the cultures after centrifugation at 200 g for 5 minutes to remove any extracapsular cells. Capsules were separated from the media before centrifugation. rIgG1 present in the media

was purified using Protein A Nab spin columns and quantified using the nanodrop at A 280 nm.

Figure 6.2.3.4.1 below allows comparison between the concentrations of rIgG1 present in the cultures over the course of the cultivation period. The operation of the controlled-fed perfusion mode of cultivation maintained the concentration of rIgG1 below the maximum concentration typically present in the batch culture. The determination of the concentration of recombinant protein in cultures throughout the culture period is important in instances where proteins may be sensitive to concentration dependent aggregation. As the rIgG1 protein produced by the CHO DP-12 cell line has not been noted to aggregate in the concentrations achieved to date, it is not expected that concentration induced aggregation of the protein would have occurred in the controlled-fed perfusion cultures, as will be verified in Section 6.2.3.5.

Figure 6.2.3.4.2 and the data presented in Table 6.2.3.4.1 allow for comparisons to be made between the cumulative total amount of IgG1 produced by cells grown in the 3 different culture modes and also the specific and volumetric productivities observed. There was no significant difference in the specific production rate displayed by the cells during the exponential growth phase when cultured under batch and control-fed perfusion modes of cultivation. The lowest mean value over this time period was in the controlled-fed perfusion culture conducted in the 5 % CO₂ incubator, however it was not significantly lower than that observed in the batch cultures. As expected, there was therefore no significant difference in the amount of IgG1 present in each culture on day 5. The application of the controlled-fed perfusion strategy to the non-CO₂ and CO₂ cultures on day 5 did result in further growth of cells until days 8 and 9 respectively. During this time period the specific productivity of the cells remained the same (non-CO₂ incubator culture) or decreased (CO₂ incubator culture) in comparison to that observed from days 1-5. It could be assumed that due to the higher number of cells present in both of the controlled-fed perfusion cultures, that the cumulative amount of IgG1 present in these cultures would be higher than the batch culture to the same degree as the increased cell numbers. Over the stationary growth period the specific productivity displayed by the cells in the control-fed perfusion (non-CO₂) and (CO₂) reduced by 36 % and 29 %, respectively. The cumulative amount of IgG1 produced in these cultures at the end of the stationary phase in the (non-CO₂) culture (day 11) and the (CO₂) culture (day 13) was 41 % and 2.65- fold higher, respectively, than that observed at the end of the stationary phase in the batch cultures (day 8). This is disappointing given the fact that there

was a 2- fold higher cell number in the non-CO₂ culture and a 5- fold higher cell number in the CO₂ during the stationary phase in comparison to the maximum cell number achieved in the batch cultures.

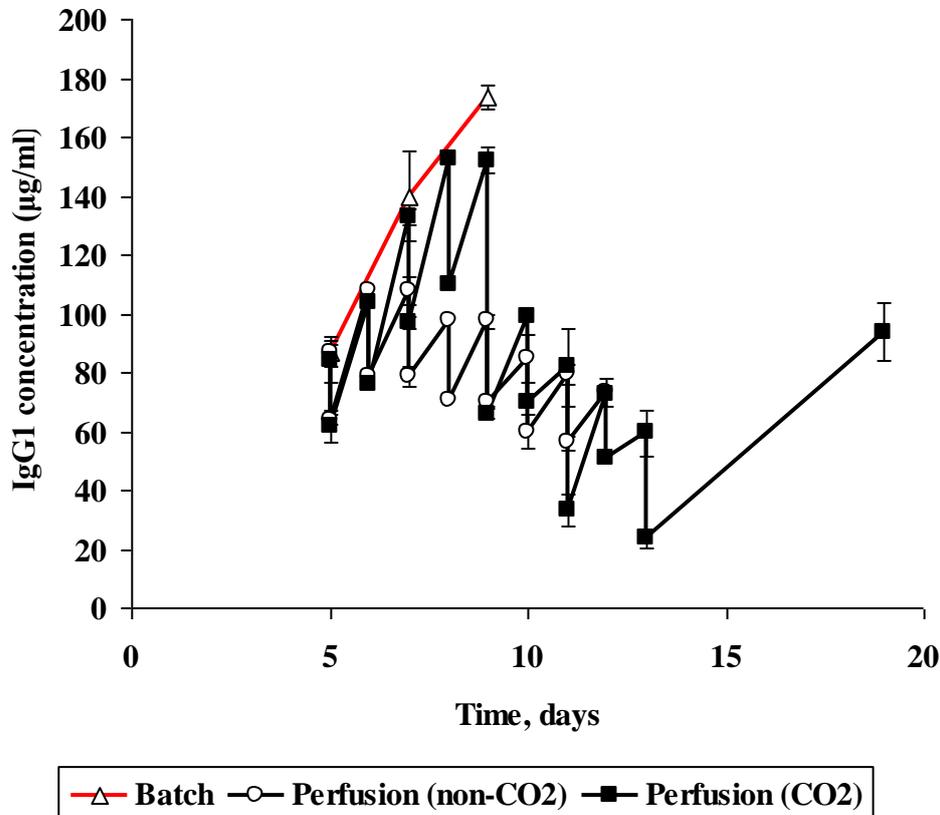


Figure 6.2.3.4.1: Concentration of rIgG1 present in encapsulated batch and controlled-fed perfusion (non-CO₂ and CO₂ incubator) cultures, performed in 1 L Erlenmeyer shake flasks, as quantified over the course of the culture period. rIgG1 present in the media was purified using commercially available protein A/G Nab spin columns and quantified using the nanodrop A280 nm. Samples were quantified in duplicate with the average value recorded. Values presented above are the average of triplicate cultures carried out in parallel, +/- STD, for each of the conditions tested.

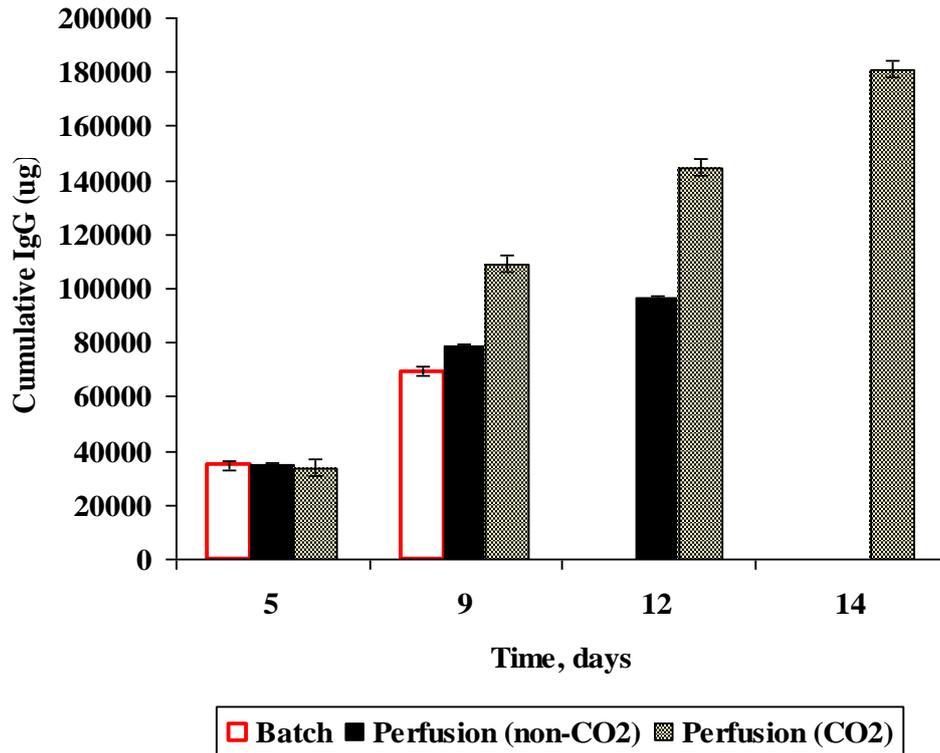


Figure 6.2.3.4.2: Total amount of IgG1 (μg) purified from cell free media samples harvested from batch and control-fed perfusion encapsulated cultures on alternating days. rIgG1 present in the media was purified using commercially available protein A/G Nab spin columns and quantified using the nanodrop A280 nm. Samples were quantified in duplicate with the average value recorded. Values presented above are the average of triplicate cultures carried out in parallel, \pm STD, for each of the conditions tested.

Table 6.2.3.4.1: Calculated rIgG1 production rates and cell specific production rates for cells encapsulated in 300 μm radius liquid capsules and cultured under batch and controlled-fed perfusion modes of cultivation in 1 L Erlenmeyer shake flasks. The volumetric production rate was calculated over the entire culture period. . Values presented are the average of those calculated for triplicate cultures carried out in parallel, \pm STD, for each of the conditions tested.

Culture	IgG1 production rate ($\mu\text{g}/\text{day}$)	q_{IgG1} ($\mu\text{g}/10^6$ cells/day) days 1-5	q_{IgG1} ($\mu\text{g}/10^6$ cells/day) 5-end growth phase	q_{IgG1} ($\mu\text{g}/10^6$ cells/day) over stationary phase
Batch	8709	2.58 \pm 0.33	NA	2.82 \pm 0.32
Perfusion (non-CO ₂)	8988.5	2.73 \pm 0.21	2.75	1.74
Perfusion (CO ₂)	15752	2.05 \pm 0.14	1.57	1.46

6.2.3.5: Product quality analysis of encapsulated cells cultured in 1L Erlenmeyer shake flasks under conditions of batch (non-CO₂ incubator), controlled-fed perfusion (non-CO₂ incubator and CO₂ incubator) modes of cultivation.

In order to assess if the application of a controlled-fed perfusion strategy would have an influence on the quality of rIgG1 harvested from encapsulated CHO DP-12 cultures, purified IgG1 samples harvested from batch and control-fed perfusion cultures were subjected to both glycan and aggregation analysis. Samples harvested from the cultures were subjected to N-glycan release and labelled as outline in Materials and Methods Section 2.3.7.1. HILIC characterisation of the released and labelled N-glycans produced a chromatogram consisting of 8 distinctly separated peaks. Each of the peaks was assigned a distinctive GU value by comparison to the retentions times of the standard A-AB labelled external dextran. Examination of exoglycosidase treated samples allowed for the corresponding number and linkage of the component monosaccharide units represented by each GU value to be elucidated, as described in Materials and Methods section 2.3.7.3. Aggregation analysis of the purified samples was carried out using SEC.

As the controlled-fed perfusion cultures essentially acted as batch cultures until day 5 of the culture period, it would be expected that there would be no significant difference in the glycoprofile obtained from IgG1 samples harvested at this point in batch and control-perfusion (non-CO₂ incubator) cultures. Any alterations in the glycoprofile present on protein harvested from control-fed perfusion (CO₂ incubator) cultures in comparison to the other two culture conditions evaluated would be due to the change in environmental condition. Analysis of Figure 6.2.3.5.1 below would suggest that there was no significant difference in the glycoprofile present on recombinant IgG1 samples harvested from the 3 different cultures on day 5.

The earliest form of N-glycosylation of rIgG1 is denoted by M5, F6A1/A2 and F6A2. The highest relative percentage of glycans present on the rIgG1 samples were the early glycoforms, predominantly F6A2. The galactosylated glycoforms are denoted by F(6)A2[6]G(4)1 and F(6)A2[3]G(4)1, which contain 1 galactose unit on either of the outer GlcNAc arms, and F(6)A2G2, which contains a galactose residue on each of the outer GlcNAc arms. Of the galactosylated glycoforms present, the F(6)A2[6]G(4)1 were the predominant form in all cultures, independent of the mode of cultivation employed

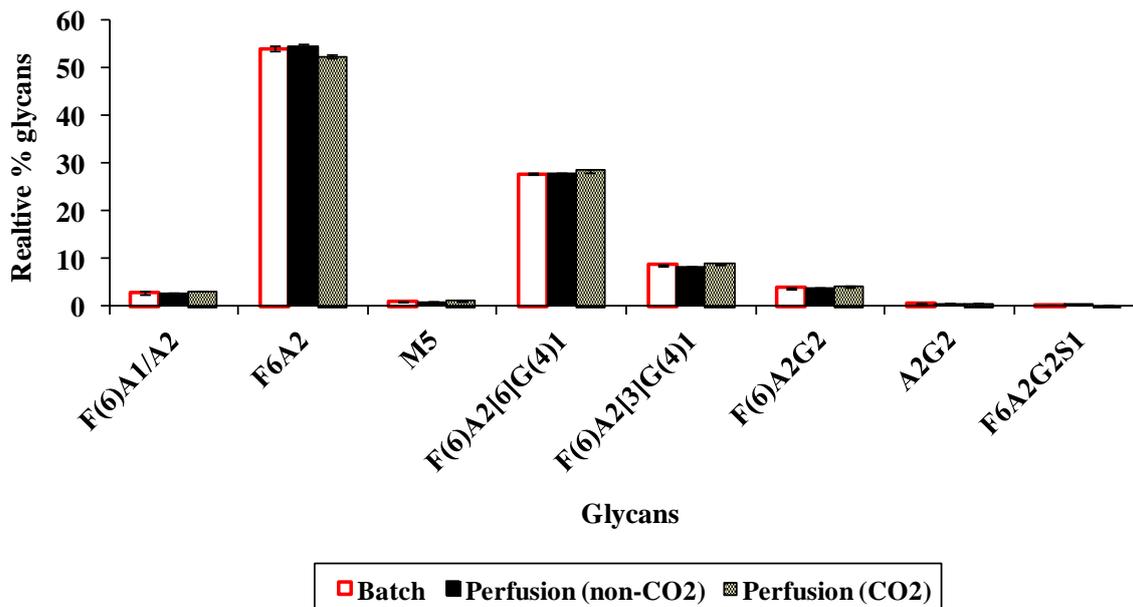


Figure 6.2.3.5.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from encapsulated cultures on day 5. Cultures were conducted in 1 L Erlenmeyer shake flasks under batch and control-fed perfusion (non-CO₂ and CO₂) modes of cultivation. N-glycans were released from the rIgG1 samples by means of PNGaseF digestion. Samples for HILIC analysis were labeled by adding 5 µl of 2AB labelling solution. The retention time of the glycan peaks was compared with an external A-AB labelled dextran standard for structural elucidation by converting the retention time of the peaks into GU. Each IgG1 sample was analysed in duplicate with the average recorded. The results are the average of the triplicate cultures (+/- STD) carried out for each condition tested.

Due to the complexity of the cultivation modes applied, it is necessary to first identify if there were changes brought about in the quality of the protein due to the application of the control-fed perfusion strategy to conditions in which encapsulated cells were cultured in a non-CO₂ environment in capped shake flasks. For this purposes Section 6.2.3.5.1 compares and contrasts the relative glycoform profile present on IgG1 samples harvested from batch and control-fed perfusion (non-CO₂ incubator) cultures. Section 6.2.3.5.2 subsequently moves forward to identify if the control-fed perfusion strategy had influences on product quality when applied to encapsulated cells cultured in a 5 % CO₂ environment. This section therefore investigates alterations in the quality of IgG1 harvested from controlled-fed perfusion non-CO₂ and CO₂ cultures at various time points.

Before analysing alterations in the glycosylation profile between the 3 culture conditions tested, it is first necessary to state that no alterations in the aggregation profile were identified for protein harvested from the 3 cultures states. Figure 6.2.3.5.1, below, illustrates the relative % of monomeric IgG1 harvested from the cultures over time.

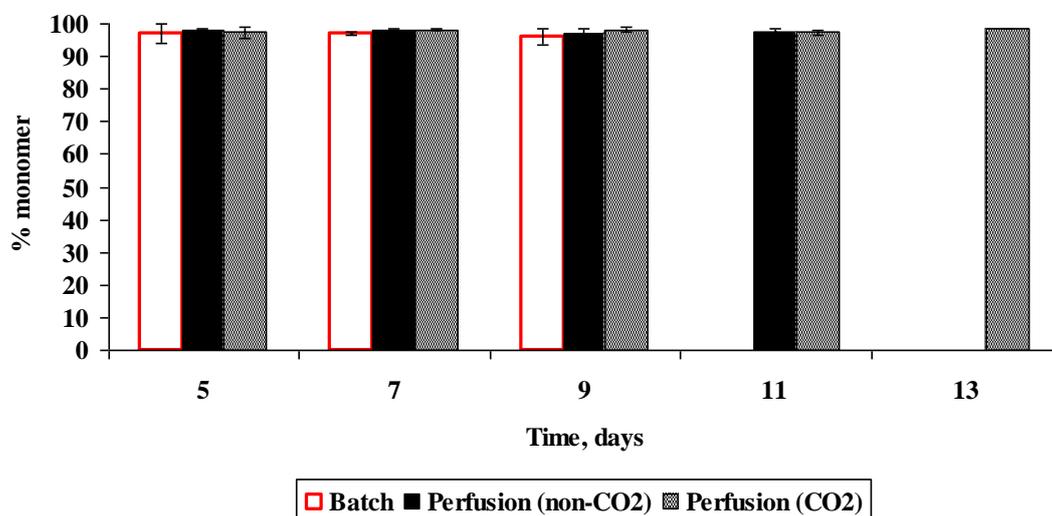


Figure 6.2.3.5.2: Relative % monomeric form of rIgG1 samples as detected by SEC. IgG1 samples were harvested from encapsulated cultures performed in 1 L Erlenmeyer shake flask under batch and control-fed perfusion (non-CO₂ and CO₂) conditions. IgG1 samples were analysed in duplicate. The results are presented as the average of triplicate cultures performed for the culture conditions tested +/- STD.

6.2.3.5.1: Batch encapsulated culture -v- Controlled-fed perfusion (non-CO₂ incubator) encapsulated culture

Both batch and control-fed perfusion cultures were carried out in a non-CO₂ incubator maintained at 37 °C. An agitation rate of 100 rpm was applied. Before any alterations in the glycosylation profile between IgG1 harvested from both culture conditions are investigated, it is firstly necessary to investigate if there were alterations in the glycan profile over the respective cultivation periods. This was already completed for the batch encapsulated cultures in Section 6.2.2.5 above. To summarise, over the course of the stationary growth period there was a decrease in non-complex glycan F(6)A2 to the degree of 12 %. The relative % of complex glycans F(6)A2[6]G(4)1, F(6)A2G2 and A2G2 were noted to increase by 42 %, 22 % and 22 % respectively. The relative % of the unprocessed glycan, M5, which is routinely noted to increase over a culture period, increased by 51 %.

Table 6.2.3.5.1.1 below gives an overview of the % increases (+) or decreases (-) noted in the relative % of specific glycan detected on IgG1 samples, over specified time period in the culture. For glycans which did increase or decrease in relative % throughout the culture period, the majority of these changes occurred in the extended growth period, from days 5-8. Over this extended growth period there was an increase in the relative % of both complex and non-complex glycans. It is important to note that the changes over time did not bring about an overall change in the specific trend of the glycans on the IgG1 protein, as noted in Figure 6.2.3.5.1 above.

Table 6.2.3.5.1.1: Overview of the % differences noted in the relative % of glycans detected on rIgG1 harvested from controlled-fed perfusion cultures (non-CO₂ incubator) at specified time point in the culture period. N-glycans were released from the rIgG1 samples by means of PNGaseF digestion. Samples for HILIC analysis were labeled by adding 5 µl of 2AB labelling solution. The retention time of the glycan peaks was compared with an external A-AB labelled dextran standard for structural elucidation by converting the retention time of the peaks into GU. Each IgG1 sample was analysed in duplicate with the average relative % of each glycan structure recorded. The results are the average of those obtained for the 3 encapsulated cultures performed under the control-fed perfusion strategy in the non-CO₂ incubator.

Glycan	% difference between day 5-8	% difference between day 8-11	% difference between day 5-11
F(6)A1/A2	+ 68 %	Not significant	+77 %
F(6)A2	-21 %	Not significant	-23 %
M5	+ 2.7 fold	+ 15 %	+ 3.1 fold
F(6)A2[6]G(4)1	+ 14 %	Not significant	+ 16 %
F(6)A2[3]G(4)1	Not significant	Not significant	Not significant
F(6)A2G2	+ 73 %	+ 11 %	+ 90 %
A2G2	+ 36 %	Not significant	+ 39 %
F6A2G2S1	Not significant	Not significant	Not significant

We have already noted in Section 6.2.3.5., above, that the relative % of glycans present on IgG1 harvested from the batch and control-fed perfusion cultures on day 5 were not significantly different. As there were changes in the relative % of individual glycans over the course of the culture periods for both conditions tested, it is therefore necessary to compare and contrast the glycoprofiles present on the IgG1 protein harvested from both cultures

towards the end of the stationary growth period, (Batch day 7 and Control-fed perfusion day 11), Figure 6.2.3.5.1.1. Recall that the only differences in the culture conditions if the controlled-fed perfusion strategy applied and the fact that such cultures reached a higher maximum cell density and degree of capsule colonisation.

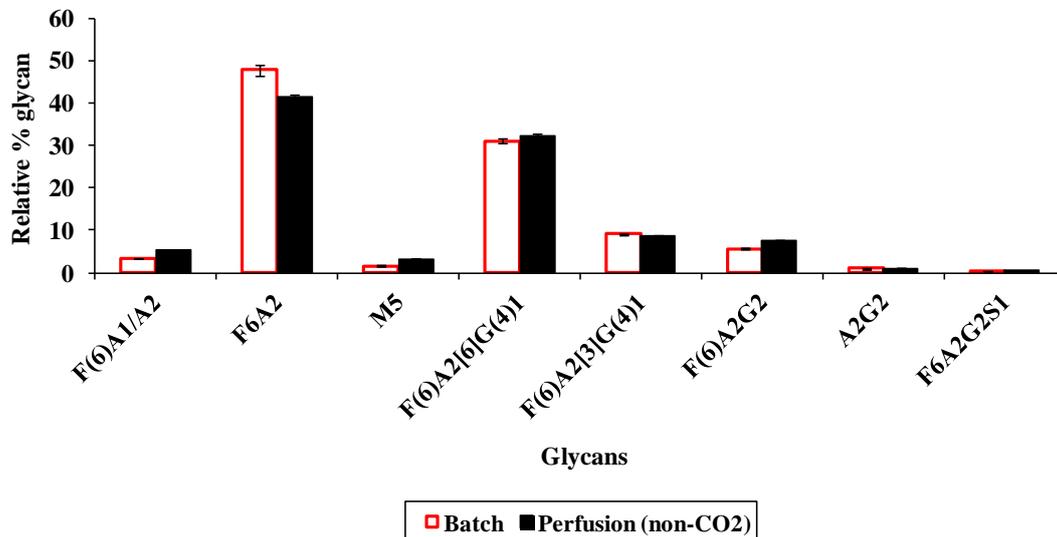


Figure 6.2.3.5.1.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from encapsulated cultures conducted in 1 L Erlenmeyer shake flask cultures under batch and control-fed perfusion modes of cultivation (non-CO₂ incubator). Samples were harvested at the end of the stationary growth period. N-glycans were released from the rIgG1 samples by means of PNGaseF digestion. Samples for HILIC analysis were labeled by adding 5 μ l of 2AB labelling solution. The retention time of the glycan peaks was compared with an external A-AB labelled dextran standard for structural elucidation by converting the retention time of the peaks into GU. Each IgG1 sample was analysed in duplicate with the average recorded. The results are the average of the triplicate cultures (+/- STD) carried out for each condition tested.

There were significant differences noted in the relative % of both non-complex and complex glycans between rIgG1 harvested at the end of the stationary growth phase for encapsulated cells cultured under batch and control-fed perfusion modes of cultivation. Of the non-complex glycans, F6A1/A2 did not change significantly over the batch culture. In control-fed perfusion (non-CO₂) culture we did see an increase of 68 % over the culture period. As both started out with similar values for F6A1/A2 at the end of the exponential phase, by the end of the stationary phase, the relative % of F6A1/A2 in perfusion (non-CO₂) was 57 % higher than

that observed in the batch. The relative % of the non-complex glycan F6A2 was 13 % higher in the batch culture, in comparison that observed at the end of the stationary phase in the control-fed perfusion culture. Based on previous studies M5 was expected to increase over the course of the culture period in both cultures. This was one of only 2 glycans which further increased in relative % over the course of the stationary phase in the control-fed perfusion (non-CO₂) culture. Over all the relative % of this glycan was over 2-fold higher than that observed in the batch culture. Of the complex glycans a slightly greater increase in the fucosylated, single arm galactosylated glycan (F(6)A2[6]G(4)1) was observed in the control-fed perfusion culture after the exponential growth period, in comparison to that which was observed in the batch culture. There was, however no significant difference in the relative % of the other fucosylated, single arm galactosylated glycan (F(6)A2[3]G(4)1) at the end of the stationary phase in both cultures. For the complex fully fucosylated and galactosylated glycan, F(6)A2G2, there was a greater increase in the relative % of this glycan occurred over the cultivation period in the control-fed perfusion (non-CO₂) in comparison to the batch culture. This resulted in a 30 % higher relative % of this glycan in the control-fed perfusion (non-CO₂) culture at the end of the stationary phase, in comparison to the batch culture. The increase in the relative % of the galactosylated but non-fucosylated glycan (A2G2) was greater in the control-fed perfusion (non-CO₂) over the culture period in comparison to the batch cultures. However this did not result in a significantly greater relative % in this glycan between the two cultures at the end of the stationary phase due to the standard deviation between the triplicate samples analysed. The most complex glycan which may be present on IgG1 harvested from the CHO cell cultures is F(6)A2G2S1. The relative % of this glycan was constant throughout the culture period in the batch and control-fed perfusion (non-CO₂) cultures.

It can be concluded that the controlled-fed perfusion culture did behave in a similar manner to the batch culture, in that the same glycans were noted to both increase and decrease in both cultures over time. The application of the controlled-fed perfusion strategy to the encapsulated cells may be considered beneficial as it did result in an overall lower relative % of non-complex F(6)A2 and a higher relative % of complex F(6)A2[6]G(4)1 and F(6)A2G2. However it must be noted that a greater increase in the relative % of M5 over the culture period was observed in the control-fed perfusion cultures in comparison to the batch cultures.

6.2.3.5.2: Controlled-fed perfusion (non-CO₂ incubator) -v- Controlled-fed perfusion (CO₂ incubator) encapsulated cultures

The main differences in parameters between the controlled-fed perfusion (non-CO₂ incubator) and the controlled-fed perfusion (CO₂ incubator) encapsulated cultures was the presence of a 5 % CO₂ atmosphere. Both cultures were incubated at 37 °C and 100 rpm agitation. The main difference observed in the behaviour of cells in the two cultures which could influence the quality of the protein produced are in the growth rate and cell specific IgG1 production rate. The control-fed perfusion culture incubated in the 5 % CO₂ incubator performed better in comparison to the other culture due to a ~50 % increased growth rate during the extended growth period (after day 5). However during the extended growth period, there was a 43 % decrease in the cell specific growth rate in the CO₂ incubated culture in comparison to the other culture. Before the glycoform profiles of the IgG1 harvested from both cultures may be compared and contrasted, it is necessary to determine also if the cultivation of the cells in the 5 % CO₂ incubator had an effect on the glycoform profile over time, Table 6.2.3.5.2.1.

Table 6.2.3.5.2.1: Overview of the % differences noted in the relative % of glycans detected on rIgG1 harvested from controlled-fed perfusion cultures (CO₂ incubator) at specified time point in the culture period. N-glycans were released from the rIgG1 samples by PNGaseF digestion. Samples for HILIC analysis were labeled with 5 µl of 2AB labelling solution. The retention time of the glycan peaks was compared with an external A-AB labelled dextran standard for structural elucidation by converting the retention time of the peaks into GU. Each IgG1 sample was analysed in duplicate with the average relative % of each glycan structure recorded. The results are the average of those obtained for the 3 encapsulated cultures performed under the control-fed perfusion strategy in the CO₂ incubator.

Glycan	% difference between day 5-9	% difference between day 9-13	% difference between day 5-13
F(6)A1/A2	+ 63 %	+ 2.09 fold	+ 3.42 fold
F(6)A2	- 21 %	+ 46 %	+ 16 %
M5	+ 2.6 fold	Not significant	+ 2.8 fold
F(6)A2[6]G(4)1	+ 13 %	- 49 %	- 43 %
F(6)A2[3]G(4)1	Not significant	- 48 %	- 47 %
F(6)A2G2	+ 59 %	-3.2 fold	- 2 fold
A2G2	+ 46 %	- 2.3 fold	- 38 %
F6A2G2S1	+ 31 %	- 68 %	- 58 %

The changes which are noted in the relative % of each type of glycan over the extended growth period in the controlled-fed perfusion (CO₂) culture (days 5-9) are almost identical to those noted over the extended growth period in the controlled-fed perfusion (non-CO₂) culture (days 5-8) (Table 6.3.3.5.1) with the exception of F(6)A2G2 which increased by 73 % in the non-CO₂ culture and only by 59 % in the CO₂ culture. Another difference may be noted is in the relative % of the fucosylated and sialylated F6A2G2S1, which was not noted to change at all in the non-CO₂ but actually increased by 31 % during the extended growth period in the CO₂ culture.

The results presented in Table 6.2.3.5.2.1 above and Figure 6.2.3.5.2.1 below would suggest that the application of the control-fed perfusion culture in a 5 % CO₂ environment did not influence the quality of the protein significantly differently at the end of the extended growth period (day 9) to that which was observed at the end of the extended growth period (day 8) in the non-CO₂ culture. In section 6.3.3.5.1 above, it was noted that the application of the controlled-fed perfusion strategy to the non-CO₂ incubated flasks was beneficial in that the relative % of more complex glycans was higher than that observed at the end of a batch culture under the same conditions. The same benefits are noted for the control-fed perfusion culture in the CO₂ incubator, with the additional benefit of a higher increase in the relative % of the fully fucosylated and sialylated F6A2G2S1 glycan. This glycan did also increase throughout the extended growth period in the non-CO₂ culture, however due to standard deviations between the triplicate cultures, the result could not be shown to be significant. There was no significant difference in the relative % of this F6A2G2S1 glycan between the two cultures at the end of the extended growth period.

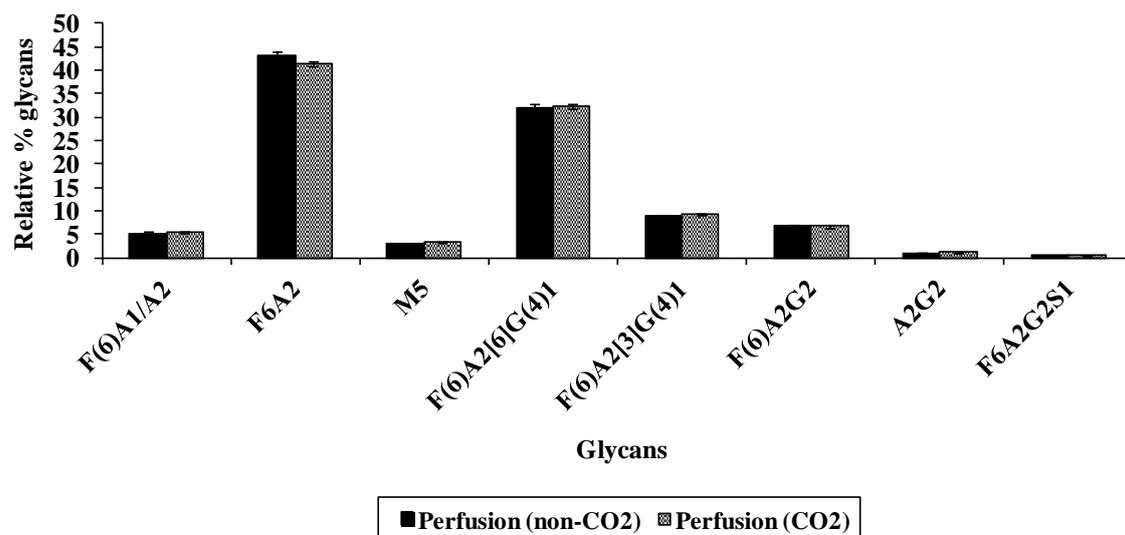


Figure 6.2.3.5.2.1: Relative % of N-glycan forms detected on rIgG1 samples harvested from encapsulated cultures conducted in 1 L Erlenmeyer shake flask cultures under control-fed perfusion modes of cultivation in both a non-CO₂ and 5 % CO₂ environment. Samples were harvested at the end of the extended growth periods, day 8 for non-CO₂ incubated cultures and day 9 for CO₂ incubated cultures. N-glycans were released from the rIgG1 samples by means of PNGaseF digestion. Samples for HILIC analysis were labelled by adding 5 µl of 2AB labelling solution. The retention time of the glycan peaks was compared with an external A-AB labelled dextran standard for structural elucidation by converting the retention time of the peaks into GU. Each IgG1 sample was analysed in duplicate with the average recorded. The results are the average of the triplicate cultures (+/- STD) carried out for each condition tested.

It was noted in Section 6.2.3.5.1 above that the majority of the changes in the relative % of glycans observed in the non-CO₂ incubated control-fed perfusion culture occurred by the end of the extended growth period on day 8. In contrast to this, significantly large changes were noted to occur in the relative % of each glycan, with the exception of M5, over the stationary growth period in the CO₂ incubated control-fed perfusion culture. Figure 6.2.3.5.2.2 below, allows for the glycoform profile present on IgG1 harvested from both control-fed perfusion cultures at the end of their respective stationary phases to be compared and contrasted.

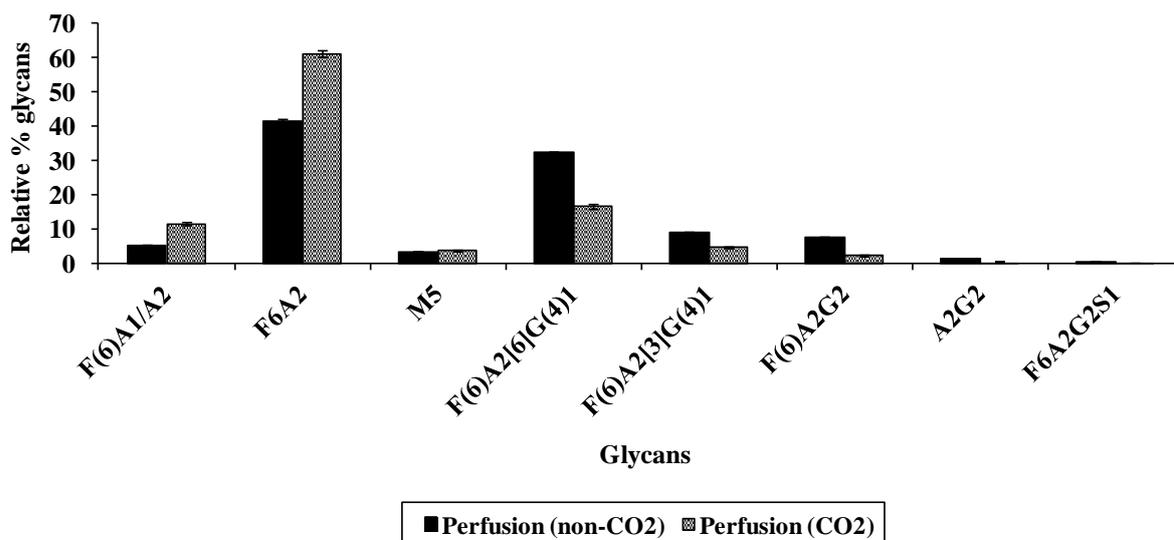


Figure 6.2.3.5.2.2: Relative % of N-glycan forms detected on rIgG1 samples harvested from encapsulated cultures conducted in 1 L Erlenmeyer shake flask cultures under control-fed perfusion modes of cultivation in both a non-CO₂ and 5 % CO₂ environment. Samples were harvested at the end of the stationary growth periods, day 11 for non-CO₂ incubated cultures and day 13 for CO₂ incubated cultures. N-glycans were released from the rIgG1 samples by means of PNGaseF digestion. Samples for HILIC analysis were labelled by adding 5 µl of 2AB labelling solution. The retention time of the glycan peaks was compared with an external A-AB labelled dextran standard for structural elucidation by converting the retention time of the peaks into GU. Each IgG1 sample was analysed in duplicate with the average recorded. The results are the average of the triplicate cultures (+/- STD) carried out for each condition tested.

For the non-complex glycans F(6)A1/A2 and F6A2 there was a 2.1 fold and 45 % increases respectively in the relative % of these glycans present on IgG1 in the CO₂ incubated cultures, in comparison to the non-CO₂ incubated cultures at the end of the stationary growth period. For the other non-complex glycan M5, there was no significant difference in the relative % of this glycan between the two culture conditions investigated. A dramatic decrease in the relative % of the complex fucosylated and single arm galactosylated glycans, F(6)A2[6]G(4)1 and F(6)A2[3]G(4)1, was noted at the end of the stationary growth period in the CO₂ incubated cultures to the degree of 49 and 48 % respectively. The glycans were therefore also 49 and 48 % lower in relative % in the CO₂ incubated culture in comparison to the non-CO₂ incubated culture also. A 3.2- fold lower relative % of the complex fucosylated and double

galactosylated glycan F6A2G2 was observed at the end of the stationary growth period in the CO₂ incubated culture, which resulted in 72 % lower relative % of this glycan at the end of the stationary phase in the CO₂ incubated culture in comparison to the non-CO₂ incubated culture. For the non-fucosylated, double galactosylated glycan A2G2, there was a 2.3- fold lower relative % of this glycan at the end of the stationary phase in comparison to the end of the extended growth period in the CO₂ culture, resulting in a 55 % lower relative % of this glycan at the end of the stationary phase in comparison to that observed in non-CO₂ cultures. For the sialyated glycan F(6)A2G2S1, there was a 68 % lower relative % of this glycan present at the end of the stationary phase in comparison to the end of the extended growth period for the CO₂ incubated culture. In comparison to the non-CO₂ incubated culture, the relative % of the glycan was 60 % lower at the end of the stationary growth periods. The large increases in non-complex coupled with the large decreases in complex for the CO₂ incubated control-fed perfusion culture suggest that this mode of cultivation had detrimental effects on protein quality. They may indicate the importance of harvest time, as the losses become more profound over the stationary phase (day 9, 11, 13 comparison) (results not shown).

6.3: Discussion:

Encapsulation of mammalian cells as a cultivation technique yielding high cell densities and high volumetric productivities has been extensively reported, as reviewed by Selimoglu, and Elibol, (2010). However the possibilities of implications on protein quality (glycosylation and/or aggregation) as a direct effect of cell cultivation in such microcapsule environments have been somewhat over looked. Any alterations in cellular growth rates (Butler, 2006), cell specific productivities (Hooker et al., 1999) or cell metabolism (Nyberg et al., 1999) as a direct result of the growth of cells in a microcapsules environment, as oppose to freely in suspension has the potential to influence the relative glycoform of the recombinant protein being produced. As higher volumetric productivities have been noted when mammalian cells are cultured in microcapsules, in comparison to that of a suspension culture mode (Brequet et al., 2007; Zhang et al., 2007; Wen-tao et al., 2005; Sinacore et al., 1989), there is also the possibility for concentration dependent aggregation. Previous work in this thesis (Chapter 5) has reported on 3.7- fold increases in maximum cell number and increased volumetric productivity which occurred when a CHO DP-12 cell line is cultured in APA microcapsules under batch cultivation conditions, in comparison to that which may be

noted when the cells are cultured in suspension. There was no great implication on the quality of the protein as a direct result of cell encapsulation. The relative % of each type of glycan was similar for protein harvested from both suspension and encapsulated cultures. There was however an increase, 2.7- fold, in the relative percentage of the non-complex, high mannose M5 glycan. No changes in M5 were noted over time in the suspension cultures. This could be indicative of a loss in complex glycan processing. However this slight increase was not coincided with a decrease in any of the complex glycans. It could therefore be concluded that the quality of the protein was not overly affected by the movement to an encapsulated mode of cultivation.

The degree of colonisation of the capsules with cells noted in Chapter 5, was calculated at ~ 5 % due to the batch nature of the culture. A feeding strategy was therefore proposed that would alleviate the nutrient limitations which occur in batch cultures and thus potentially increase the degree of colonisation of the microcapsules. Perfusion as a mode of cultivation yielding increased viable cell numbers and volumetric recombinant protein production in comparison to those usually attained in a batch culture has been extensively reported for cells cultivated in suspension (Hu et al., 2011; Ryll et al., 2000; Meuwly et al., 2006; Link et al., 2004; Leelavatcharamas et al., 1999). Suspension cells are retained inside the bioreactor usually by means of filtration or sedimentation, as reviewed by Voisard et al., (2003). Perfusion modes of cultivation have however also been applied to cells immobilised/entrapped on or in some form of 3-D structure such as microcarriers and immobilised within microcapsules which protects them from the external environment and prevents the cells from being washed out of the cultivation vessel during the removal of media. Increased cell yields and volumetric productivities have also been reported for such applications in comparison to batch or fed-batch cultivation techniques (Kong et al., 1998; Lee et al., 2005; Wang et al., 2002; Goldman et al., 1998; Seifert and Philips, 1997). Although the overall aim of this current study was to investigate if the % colonisation of the capsules could be increased, the effect of the feeding strategy on all cellular activities (growth, productivity, metabolism and product quality) would be determined. A control-fed perfusion strategy was designed in which the perfusion rate would remain constant, unless a high accumulation of by-products (lactate or ammonia) were noted at a later stage in the culture. The concentration of the nutrients glucose and glutamine would be forward adjusted in the feed media to meet the nutrient demands of the cell numbers predicted to be present in the culture on the following day. Such predictions were made based on cell growth and

specific consumption rate data accumulated. Other control-fed perfusion cultures have been designed similarly in which the concentration of particular nutrients in the media are altered to meet the requirements of a forward predicted cell number for the following day (Jain and Kumar, 2008), however the perfusion rate may also have been adjusted. Yang et al., (2000), reported on 8- and 1.8- fold increase in maximum viable cell numbers achieved for an Sp2/0-Ag 14 cell line when cultured under a control-fed perfusion strategy in comparison to that observed utilising batch and perfusion modes of cultivation respectively. Teng et al., (2011) reported a 3.3- fold increase in maximum viable cell number under a control-fed perfusion strategy in comparison to that achieved in batch cultures. Such cultures were carried out when cells were in suspension. The potential for a similar-control fed perfusion strategy to lead to increased cell yields and volumetric productivities was determined for encapsulated cell cultures.

Initial studies were conducted in the same 1.7 L minifors reactor platform that was utilised for the batch encapsulated studies in Chapter 5. The control-fed perfusion strategy was applied to the culture on day 5, when glucose and glutamine concentrations were insufficient to support the growth of the predicted number of cells which would be present on day 6. The study was successful in that the $\text{cells/ml}_{\text{capsules}}$ did continue to increase on a daily basis until day 9. The degree of colonisation at maximum cell number ($\text{cells/ml}_{\text{capsule}}$) on day 8 was noted to be 9.3 %. This was therefore an 86 % increase in capsule colonisation in comparison to batch cultures. This increased cell number per $\text{ml}_{\text{capsules}}$ did not however translate into an overall higher yield in $\text{cells/ml}_{\text{reactor}}$ in comparison to the batch cultures. An instability in the capsules was noted in the control-fed perfusion culture, perhaps due to an increase cell numbers present, or the changing of media on a daily basis, which resulted in a loss of capsules and subsequent cell wash out as the capsules provided the only mechanism for cell retention in the perfusion system. Brequet et al., (2007) reported that the perfusion nature of a culture further reduces the stability of the already relatively fragile liquid core microcapsules. They reported that in a bioreactor, a combination of shearing forces and a constant displacement of equilibrium between the fresh perfusion medium and the microcapsules, may be destructive for liquid core APA microcapsules.

The potential of this initial study for high cell density culture was limited by the capsule instability. It did however give an indication of cellular behaviour when cell colonisation inside the microcapsules is increased. There were no changes in the specific productivity of the cells due to increased colonisation or the application of a feeding strategy eliminating

nutrient limitations. The volumetric productivity was similar to that of the batch culture throughout the entire culture period and as there was no significant difference in $\text{cells/ml}_{\text{capsules}}$ in the cultures, the specific productivity was not influenced. The main difference between the batch and control-fed perfusion cultures was a lack of depletion of glucose and glutamine concentrations throughout the culture period and also the removal of spent media thus reducing by-product accumulation. After day 5, the lactate production rate was noted to be slightly higher than that observed in the batch cultures, resulting in an increased yield of lactate on glucose by 27 % in comparison to the batch cultures. This was attributed to the fact that the cells were essentially over-fed with glucose as a direct result of the over-prediction of expected cell numbers which would be present the next day. This statement is verified by the fact that it has been reported that at low glucose concentrations, glucose is used primarily to produce anabolic precursors such as nucleotides, while at higher concentrations most of the glucose enters glycolysis and results in pyruvate formation, much of which forms lactate (Banik and Heath, 1995). The maximum lactate concentrations associated with batch cultures is essentially ~ 17 mM. However the concentration of lactate present in the control-fed perfusion culture approached ~ 40 mM by the end of the culture period. This concentration of lactate is not considered growth limiting for CHO (Lao and Toth, 1997) or other mammalian cell lines such as hybridoma cells (Zhang et al., 2004).

The specific glutamine and ammonia consumption and production rates remained similar throughout the duration of the control-fed perfusion culture. The maximum concentration of ammonia present in the perfusion culture was similar to that of the batch cultures, 4.3 and 4.8 mM respectively. In regards to product quality, the control-fed perfusion culture did give indications that the large increases in M5, ~ 2.8 -fold, which are observed to occur in batch cultures over time could be reduced. It was explained for the batch culture in Chapter 5, that the increase in M5 over the stationary growth period was as a direct result of a glutamine limitation over this time period. An evaluation of the glutamine;fructose-6-P amidotransferase (GFAT) network for Uridine Di Phosphate-N-acetylhexosamines (UDP-GNac) synthesis (Nyberg et al., 1999) demonstrates how a limitation in glutamine results in decreased Glucosamine-6-phosphate and subsequently lower levels of N-Acetylglucosamine 1-P. This N-Acetylglucosamine 1-P utilises uridine triphosphate (UTP), which is sensitive to glucose limitations, for the formation of the nucleotide sugar precursors UDP-GNac's. It may be recalled that in the batch culture a limitation in glutamine at the end of the exponential growth phase occurred. Glucose did not become limiting until later in the

stationary phase (day 7). Therefore a limitation in UDP-GNAc may have occurred in batch cultures due to a limitation in N-Acetylglucosamine 1-P. A depletion of UDP-GNAc prevents the processing of M5 into more complex glycans containing N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) residues. The relative % of M5 is quite low in comparison to all other glycans in general. On a positive note, the increase in M5 over the batch culture period did not significantly reduce the relative % of the more complex glycans which are usually present at high levels.

A 32 % increase in M5 occurred over the culture period in the control-fed perfusion culture, significantly lower than the 2.7-fold increase noted to have occurred in the batch cultures. This is due to the fact that glutamine was not depleted at any stage in the control cultures. The control-fed perfusion culture did also give an indication that the relative % of the complex fucosylated and sialylated glycan could be increased over time, due to the maintenance of key metabolites. There was an 80 % increase in F(6)A2G2S1 over the culture period in the control-fed perfusion culture. No increases in this glycan were noted over the batch encapsulated culture period. It must however be noted that the perfusion culture was only carried out once. It is therefore difficult to interpret if the increase in F(6)A2G2S1 is of any significance. A possible explanation for an increase in sialylation would be the presence of glutamine throughout the entire culture period in the control-fed perfusion culture. We have seen above from an evaluation of the GFAT network that glutamine is necessary for maintaining an intracellular concentration of UDP-GNAc's such as, uridine-N-acetylglucosamine (UDPGlcNAc) and uridine-N-acetylgalactosamine (UDP-GalNAc). UDP-GlcNAc has a role to play in sialylation as it is required for the formation of ManNAc which is a precursor for the sialic acid transporter donor Cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-NANA) (Burleigh et al., 2011, Butler 2006) The prevalence of glutamine in the control-fed perfusion culture may therefore be responsible for an increase in sialylation over time. It must be noted however, that M5 did increase throughout the culture. This may be due to the fact that although UDP-GNAc were not limiting, they may have been utilised to some extent in the formation of ManNAc leading to increases in sialylation. However it is also important to be aware of the fact that it has been noted that UDP-GNAc are favourably used in the formation of biantennary glycan structures and that sialylation is considered as the last step in glycan processing and possibly more sensitive to a reduction in UDP-GNAc's than the formation of biantennary structures could be (Wong et al., 2004). It must be noted that the increases in M5 were not of an extreme nature in the control-fed

perfusion culture and in this sense sialylation did increase due to the availability of UDP-GlcNAc brought about by the lack of glutamine depletion.

Disregarding the limitations in the microcapsules as effective retention devices for a perfusion system, it may be concluded that the control-fed perfusion strategy has been successful in allowing for enhanced capsule colonisation coupled with possible enhancements in product quality over time.

Following the investigation of a control-fed perfusion strategy on microcapsule colonisation, there were two possible steps forward identified: (I) investigation of the production of microcapsules with increased stability properties or (II) maintain the utilisation of the APA microcapsules with transfer of the control-fed perfusion culture to the less harsh 1 L Erlenmeyer shake flask platform. Liquid core APA microcapsules are renowned for relatively poor stability properties. This is attributed to the removal of calcium ions from the core as a direct result of the final washing step with sodium citrate (typically 0.05 M). Elimination of this step results in what are known as semi-liquid core microcapsules. Although they provide space limitations for capsule colonisation, due to presence of solid alginate pieces in the core of the capsules, they have been noted to display enhanced strength properties for use in mammalian cell cultures (Brequet et al., 2007). Various authors have investigated mechanisms for increased liquid core APA capsule strength including: coating the capsules with alginate of higher molecular weight (Koch et al., 2003); increased number of PLL layers (Koch et al., 2003; Leick et al., 2011); higher concentrations of PLL for increased crosslinking with alginate for thicker membranes (Koch et al., 2003); reduced capsule sizes (Zhang et al., 2007); and more novel methods utilising different forms of PLL (poly-D-lysine, poly-L-ornithine, ϵ -PLL) (Castro et al., 2005; Ma et al., 2012) or the inclusion of vinyl monomers and a photoinitiator allows to diffuse into pre-formed calcium alginate beads (Wang et al., 2005). The results however have been varied in the significance of improvements in capsule stability over time (Castro et al., 2005).

In order for the work to progress and for a full analysis of the effects of both increased colonisation and the application of a controlled-fed perfusion mode of cultivation on recombinant protein quality, the work progressed with option (II). As the encapsulated CHO DP-12 cell line had not yet been characterised under a batch mode of cultivation in a 1 L Erlenmeyer shake flask platform, it was first necessary to investigate the comparability of the

activities of encapsulated cells in the 1.7L minifors and 1 L Erlenmeyer shake flask platform. In this way any alterations which would occur in encapsulated cell activities after application of the control-fed perfusion strategy to the 1 L Erlenmeyer shake flask could be concluded to be as a direct effect of the application of the feeding strategy and not due to the scale down of the cultures. In Chapter 4, alterations in cellular activities were reported in the scale-up of suspension cells.

No significant difference in cellular growth rates or maximum viable cell number attained ($\text{cells/ml}_{\text{capsules}}$ and $\text{cells/ml}_{\text{reactor}}$) were noted between encapsulated cells cultured in both 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms. Cell specific production rates and maximum IgG1 concentrations reached were comparable between both culture platforms. Cell specific and volumetric rates of glucose and glutamine consumption and lactate production were also comparable between the two culture platforms. There was a reduction in the specific ammonia production rate in the 1 L Erlenmeyer shake flask platform in comparison to the 1.7 L minifors reactor cultures. This resulted in an overall reduced maximum ammonia concentration of 31 % in the 1 L Erlenmeyer shake flask cultures, which gave rise to a reduction in the yield of ammonia on glutamine of 35 %. As well as alterations in ammonia production, there were alterations in the quality of the protein harvested from the 1 L Erlenmeyer shake flask cultures in comparison to the 1.7 L minifors reactor cultures. These are related to changes in the glycans occurring over time in the 1 L Erlenmeyer shake flask culture, as no differences were noted in the glycoform profile of IgG1 harvested from cultures performed at the end of the exponential phase in both platforms.

There were changes in the relative % of 4 of 8 the glycans detected on IgG1 harvested from 1 L Erlenmeyer shake flask cultures at the end of the exponential and stationary growth phases. Between the two growth phases there was a 12 % decrease in the non-complex F(6)A2 glycan, a 42 % and 22 % increase in the relative % of the complex glycans F(6)A2[6]G(4)1 and F(6)A2G2, respectively, and a 22 % increase in the relative % of A2G2. The only glycan noted to increase over time in the encapsulated cultures performed in the 1.7 L minifors reactor was the M5 glycan, which increased by 2.7-fold. M5 did also increase over the culture period in the 1L Erlenmeyer shake flask culture, however by the smaller degree of 51 %.

The main aim of the study was not to investigate the alterations in the glycosylation profile of recombinant IgG1 overtime when harvested from encapsulated cultures performed in a 1 L Erlenmeyer shake flask. Although the findings are of interest, the study sought to determine if the quality of the protein harvested at the end of the culture period from encapsulated cultures

performed in 1 L Erlenmeyer shake flasks was comparable to that harvested from encapsulated cultures performed in 1.7 L Erlenmeyer shake flasks. The relative % of M5 glycan was 39 % lower at the end of the stationary phase in 1 L Erlenmeyer shake flask cultures in comparison to 1.7 L minifors reactor cultures. The relative % of the glycan F(6)A2G2S1 was 53 % and 63 % higher in the 1 L Erlenmeyer shake flask culture at the end of the exponential and stationary growth phases, respectively.

Heterogeneity studies investigating alterations in recombinant glycoprotein glycosylation during a cultivation period have been examined by various authors. An investigation by Wong et al., 2010 focused on the up- and down-regulation of 21 different glycosylation genes as a possible cause for reduced sialylation levels present interferon- γ (IFN- γ) over the course of a fed-batch culture. Various authors have investigated the impact of limiting glucose and glutamine concentrations on glycan heterogeneity throughout a culture period (Hooker et al., 1995; Xie et al., 1997; (Wong et al., 2005). Cell viability and the subsequent release of glycosidase enzymes as a result of cell apoptosis have also been noted to be responsible for changes in glycan heterogeneity over a culture period (Wong 2005; Yang and Butler 2000; Xie et al., 1997). All of these studies report on decreases in the relative % of specific glycans over time in a culture. This current study has identified increases in the relative % of glycans over time for IgG1 harvested from encapsulated cell cultures performed in 1 L Erlenmeyer shake flask platforms.

A possible reason for a lower increase of M5 over time in the 1 L Erlenmeyer shake flask culture in comparison to the 1.7 L minifors reactor culture are linked to the fact that in the 1 L Erlenmeyer shake flask encapsulated culture, glucose was not completely distinguished at the end of the stationary growth period. On day 8 of the culture period, the glucose concentration was in the range of 1.5 mM in the shake flask culture. In the encapsulated minifors reactor culture, all of the glucose was depleted at the end of day 7. Banik and Heath (1995) had stated that when glucose is limiting it may be utilised for nucleotide sugar synthesis (i.e. anabolic reactions as oppose to catabolism). It is therefore possible to suggest that the availability (low concentration) of glucose in the 1 L Erlenmeyer shake flask culture would have resulted in a higher intracellular pool of UTP, a precursor for the formation of UDP-GlcNAc, which is required for the transfer of GlcNAc and GalNAc residues onto the emerging glycan chain on the recombinant protein.(This could not occur in the 1.7 L Erlenmeyer shake flask culture as the glucose was not limiting but rather depleted by day 7). Therefore a decreased accumulation of M5 glycan, coupled with an increasing relative % of

more complex glycans over time may have been attributed to the availability of glucose in the 1 L Erlenmeyer shake flask culture right up until the culture was ceased.

It is important to note that glutamine was limited in both the shake flask and minifors reactor cultures after the exponential growth period. In the GFAT network glutamine is required for the formation of N-Acetylglucosamine 1-P which together with UTP forms the UDP-GNac nucleotide sugars. This statement therefore assumes that the intracellular content of N-Acetylglucosamine 1-P was sufficient after the glutamine was depleted. It is clear that glutamine metabolism was of an efficient nature during the exponential growth phase, noted by the decrease in the specific ammonia production rate the 1 L Erlenmeyer shake flask culture in comparison to the 1.7 L minifors reactor culture. The increases in sialylation noted in the 1 L Erlenmeyer shake flask encapsulated culture and that of the 1.7 L minifors reactor culture may also be due to the increased levels of UDP-GNac present in the cells cultured in the shake flasks due to the availability of glucose. As noted above, UDP-GlcNAc is utilised to some extent in the formation of ManNAc leading to increases in sialylation. The theory used to suggest that the prevalence of a low glucose concentration in the 1 L shake flasks in comparison to the 1.7 L minifors reactor cultures is responsible for lower levels of M5 glycan in the shake flask culture is verified by statements made in Chapter 4 in which differences in quality between two different scales of culture could be attributed to altered metabolism at these scales.

Although alterations were noted in the glycosylation profile of IgG1 harvested from batch encapsulated cultures performed in 1 L Erlenmeyer shake flasks and 1.7 L minifors reactor cultures, the main aim of the study was to also to determine if the 1 L shake flask platform holds potential for increased microcapsules stability suitable for the application of a control-fed perfusion culture and increased culture longevity. Analysis of the mechanical resistance data obtained from Texture Analyser analysis of capsules from both conditions did indicate that there was a similar decreasing trend in mechanical resistance, irrelevant of the culture platform used. However, the time taken for the microcapsules in 1 L Erlenmeyer shake flasks to reach the same low level of mechanical resistance, 0.07 g/capsule, displayed in 1.7 L minifors reactors was lengthened by ~ 2 days. Ideally it would have been optimal if the mechanical resistance of microcapsules in shake flasks did not reach such a low level at any stage during the culture period. However it may be hypothesised that the less harsh conditions provided by a shake flask may allow the capsule to maintain integrity for the duration of a control-fed perfusion culture.

Having compared and contrasted all encapsulated cellular activities between 1 L Erlenmeyer shake flask and 1.7 L minifors reactor platforms, it was then necessary to go back to the original aims of the work. An investigation was conducted as to whether the control-fed perfusion strategy could lead to increased capsule colonisation, resulting in real increases in viable cell number per $\text{ml}_{\text{vessel}}$ for encapsulated cells cultured in the 1 L Erlenmeyer shake flask culture. From the initial control-fed perfusion studies performed in the 1.7 L minifors reactor, increased capsule colonisation was possible. Control-fed perfusion cultures have not been applied to cells cultured in microcapsules to the best of our knowledge. However studies carried out with CHO cells in suspension have noted on the beneficial effects of such a strategy of perfusion in relation to increased cell numbers being attained (Teng et al., 2011; Yang et al., 2000). The control-fed perfusion strategy was first applied to encapsulated cells cultured in non-vented shake flasks as this is directly comparable to the batch encapsulated shake flask cultures. The application of the control-fed perfusion strategy to these encapsulated culture, termed “control-fed perfusion non-CO₂ cultures”, resulted in a < 2-fold increase in capsule colonisation with a capsule colonisation of over 11 % at maximum viable cell density. Exponential growth at the maximum specific growth rate typically ceased on day 5 of an encapsulated batch culture. The increase in capsule colonisation noted in the control-fed perfusion non-CO₂ culture was due to an extended growth period beyond day 5 in which the growth rate was determined to be half the value of the maximum growth rate observed during the exponential growth period (days 1-5). This extended growth period ended on day 8 and was possibly linked to a lack of aeration for these higher cell densities due to the unvented nature of the shake flasks. In perfusion cultures employing full cell retention systems it has been reported how when all other parameters are optimal, the only factor which may limit the cell density may be the level of dissolved oxygen (Hiller et al., 1993). The control-fed perfusion strategy applied in this study caused what are known as “pulse additions” of substrates resulting in subsequent “pulse” increases in metabolite concentration. Such a mode of cultivation has been noted to cause increases in the specific oxygen consumption rate displayed by hybridoma cells (Hiller et al., 1993). Increased metabolite concentrations usually result in increased TCA cycle activity and thus an increased oxygen uptake rate (OUR) (Banik and Heath, 1995). The available oxygen may have been limited in the non-vented shake flask studies. Glucose and glutamine were not limiting during the point at which the extended growth period ended and a stationary growth

phase commenced. The fact that the cells were growing at a lower rate in the extended growth period than that observed in the exponential phase may indicate that there was an aeration limitation. The control-fed perfusion strategy did perform well in the prevention of a depletion of the two main metabolites over the cultivation period.

Due to the possibility of low mass transfer of air limiting the control-fed culture, the experiment was repeated, in a 5 % CO₂ incubator in vented shake flasks. The overall result was again an extended growth period from the time at which perfusion started (day 5) until day 9. In order to confirm that the extended growth period was due to the feeding strategy applied, a batch culture was later carried out in the 5 % CO₂ incubator under similar conditions (data not shown). The results of the batch study did confirm a cessation in cell growth on day 5, and all growth characteristics were similar to that observed for the batch cultures carried out in the non-CO₂ incubator. For the control-fed perfusion culture carried out in the CO₂ incubator, the maximum exponential growth rate was maintained in the extended growth period and the degree of capsule colonisation was determined to be 27 %. The maximum viable cell density cells/ml_{reactor} was 5- fold higher and 2.5- fold higher than that observed in the batch and control-fed perfusion non-CO₂ cultures, respectively. Increased cell yields have been reported through the application of similar feeding strategies to mammalian cell lines. Yang et al., (2000) reported a 8- and 1.8- fold increase in maximum viable cell density achieved under a control-fed perfusion strategy in comparison to cells cultured under batch and perfusion modes for an Sp2/0-Ag 14 cell line. Increased cell yields brought about by increased culture longevity as oppose to increases in cellular growth rate are consistent with those observed by others in the application of control-fed perfusion strategies. Teng et al., (2011) applied a control-fed perfusion strategy to a CHO cell line culture and reported a 3.3- fold increase in maximum viable cell density achieved in comparison to batch cultures. The increased cell yield, in the study by Teng et al., (2011), was not due to an increase in the specific growth rate displayed by the cells. The increases in cells/ml_{capsule} did translate into cells/ml_{vessel} with negligible capsule breakup being observed over the 12 day control-fed perfusion non-CO₂ and 14 day control-fed perfusion CO₂ culture period. The strength of the microcapsules was not influenced by the application of the perfusion strategy. Brequet et al., (2007) did report how the operation of a perfusion mode of cultivation may further compromise the mechanical resistance of already fragile liquid core APA microcapsules by the necessity for the capsules to constantly equilibrate with the fresh medium being added to the culture. The microcapsules did reach the low mechanical

resistance level (0.07 g/capsule) observed in other encapsulated studies in both minifors reactors and shake flasks. However, due to the less harsh shear environment of the shake flasks, the capsules did remain intact for the duration of the control-fed perfusion cultures in the 1 L Erlenmeyer shake flasks.

The cellular growth patterns which were noted in the two control-fed perfusion cultures may be characterised into 4 distinct phases, as explained by Breguet et al., (2007). In the study a CHO cell line was cultured in APA liquid core microcapsules under a perfusion mode of operation. The growth of the encapsulated cells followed the pattern of (1) an exponential growth phase during the time period which the culture operated as a batch culture (i.e. before perfusion is initiated) (2) a second semi-exponential growth phase with continuous medium supply (3) a stationary phase (4) a death phase. This is the typical growth pattern of encapsulated cells under conditions of a constant perfusion rate with full retention (Gugerli, 2003). The perfusion rate in this current control-fed perfusion study also remained constant over the growth periods. These observed results suggest that the fortification of the perfusion medium with nutrients as required by the cells did not have an effect on the growth pattern which is typically displayed by cells which are cultured under a constant perfusion rate with full cell retention. Gugerli (2003) described the advantages of using microcapsules as effective retention devices for a perfusion system due to the principle of space limitation. It was understood that if the entire capsules were to become colonised, a feasible mechanism for maintaining cell numbers constant and achieving steady state would essentially be provided. However under conditions of full cell retention maintenance of steady state is difficult since mammalian cells follow a grow/die cycles, especially at 37 °C. This would explain the rapid decline in cell numbers present after the stationary period. In order to avoid the negative effects of the grow/die cell cycle in a perfusion culture other studies have investigated coupling a biphasic approach to a perfusion culture. When the maximum viable cell density is achieved, the culture temperature is essentially lowered to 33 °C in order to slow down cell growth, apoptosis, and nutrient uptake and to reach a steady state without cell bleeding (Ryll et al., 2000). It has been reported that in perfusion cultures, an abrupt fall in CHO cell viability typically occurs, which may not be attributed to the exhaustion of any essential amino acid or concentration of a by-product. Altamirano and Gòdia (2001) investigated the addition of a concentrated perfusion medium with the hypothesis that the inevitable onset of cell death could be avoided. The untimely onset of cell death was not in this case avoided. A possible explanation could be a limitation in oxygen due to the low

aeration capacity of shake flask environments. Mammalian cells have relatively low demands for oxygen and consequently require lower specific power input, this being typically between 1-10 Wm⁻³ (Zhang et al., 2010). However typical cell densities achieved in shake flasks are in the range of 2.5-4 * 10⁶ cells/ml (Muller et al., 2004) when conditions have been optimised. It is therefore possible that the high density cultures performed in shake flasks, both non-vented and vented, would have eventually become limited by a lack of air.

In any case, when cells are grown at high specific growth rates, a limiting condition will be reached, which is why the biphasic approach (Ryll et al., 2000) would be a favourable step forward for these control-fed perfusion cultures in shake flask. When perfusion cultures are operated under conditions of no cell bleeding, cell viability does become a limiting factor as under steady state conditions the cellular growth rate will equal the cell death rate. In a review investigating optimal bleed rate for a processes, emphasis was placed on investigating the importance of cell viability on the recombinant protein production and quality. If low viabilities should be avoided then the bleed rate should be chosen higher (Chon and Zarbis-Papastoitsis).

Due to the increased cell numbers (cells/ml_{reactor}) in the control-fed perfusion cultures, it would be expected that increases in volumetric productivity to the same degree would be achieved over the culture periods. The volumetric productivity was higher in the two control-fed perfusion cultures than the batch culture, however not to the same degree as the cell numbers. Over the extended growth period, the specific production rate remained unchanged in the non-CO₂ culture. The specific production rate was however was found to decrease by 23 % over the extended growth period in the CO₂ culture. Over the stationary growth period the specific productivity displayed by the cells in the control-fed perfusion (non-CO₂) and (CO₂) reduced by 36 % and 29 %, respectively, in comparison to that observed over the exponential growth period. The cumulative amount of IgG1 produced in these cultures at the end of the stationary phase in the (non-CO₂) culture (day 11) and the (CO₂) culture (day 13) was 41 % and 2.65- fold higher, respectively, than that observed at the end of the stationary phase in the batch cultures (day 8), indicating that overall the application of the control-fed perfusion strategies was successful in achieving higher recombinant protein in total. These increases in volumetric productivities were however lower than expected given the fact that there was a 2- fold higher cell number in the non-CO₂ culture and a 5- fold higher cell number in the CO₂ during the stationary phase in comparison to the maximum cell number achieved in the batch cultures. Studies which have previously applied control-fed perfusion

feeding strategies have reported on increased volumetric production of recombinant proteins. Yang et al., (2000) reported on an increased mAb production in the range of 10- and 2.5- fold higher than that observed in batch and perfusion cultures in comparison to the control-fed perfusion culture. Teng et al., (2011) reported on a 2.24- fold increase in volumetric productivity in a control-fed perfusion culture in comparison to a batch culture. It is typical for the concentration of a protein in a perfusion culture to remain lower than the concentration typically found in batch or fed-batch cultures under the same conditions (Hu et al., 2011). This is however due to the overflow of medium during spent medium removal. Ryll et al., (2000) reported protein concentrations in a CHO cell perfusion culture to be 20 to 80 % that observed in a batch or fed-batch processes. However the overall yield of recombinant protein was 2- to 12-fold higher than that observed in batch or fed-batch processes. Seifert and Philips (1999) have reported on increases in specific protein production when immobilised hybridoma cells are cultured under perfusion mode of cultivation in comparison to a batch mode. Goldman et al., (1998) reported that for CHO cells immobilised on microcarriers and cultured under a perfusion mode of cultivation, specific IFN- γ production was double that observed in batch cultures. Wang et al., (2002) have reported an increased specific EPO production rate for cells cultured immobilised on microcarriers under a perfusion mode of cultivation in comparison to batch or continuous cultures. Increasing EPO specific productivity in the perfusion culture did eventually level off and become stable. However none of these authors have reported on a decreased specific recombinant protein production rate. Kong et al., (1998) is only of the only studies noted to report no alterations in the specific productivity between batch and perfusion cultures using CHO cells immobilised on microcarriers. In the discussion section in Chapter 5 it was reported how in many instances cell immobilisation may induce increases specific productivities in the cells due to a number of factors to consider; stress associated enhanced specific growth brought about by impurities in encapsulation polymers (Selimoglu and Elibol 2010), initial stresses brought about by the immobilisation procedure (Selimoglu et al., 2011), a reduced growth rate enhancing production stability (Yamaguchi et al., 1997), and higher local concentrations of growth stimulating endocrine factors, (Lee et al., 1993). Even low dissolved oxygen concentrations due to mass transfer limitations in microcapsules may bring about circumstances for enhanced specific production (Ray et al., 1990). A plausible explanation for the reduced specific consumption rate across the extended growth phase in the CO₂ culture and the stationary growth phase in both the CO₂ and non-CO₂ cultures cannot be

attributed solely to the increased colonisation of the microcapsules. Firstly it is generally assumed that increased concentrations of cells in the capsules would result in locally higher concentrations of autocrine factors stimulating productivity. There was also no decrease in the specific productivity noted when increased capsule colonisation was achieved in the 1.7 L minifors reactor platform through the application of the control-fed perfusion strategy. It is more so possible that the perfusion rate was not at a sufficient level to support both growth and productivity of high cell numbers/ml_{vessel}. Commercially available cell culture media usually consists of over 30 essential nutrients, vitamins etc (Xie and Wang 2006). Over the course of the perfusion cultures it was necessary to increase the concentration of glucose to a level higher than that which is typically present in the medium. It is also possible that the concentration of other nutrients in the medium was not sufficient in the volume of medium used to perform the perfusion, and so cell performance at the production level was compromised. Meuwly et al., (2004) did see alterations in the volumetric productivity of in a CHO cell culture operated under a perfusion mode of cultivation in a packed bed bioreactor. Volumetric productivity was noted to decrease with decreasing perfusion rates. Yamaguchi et al., (1997) does state that the high specific productivities are simply brought about by adjusting the culture conditions so that they can be maintained at the optimum conditions. Low oxygen levels, beyond the requirements of the cells, may also a possible cause for a loss in specific productivity (Link et al., 2004). It was stated above how a possible aeration limitation was responsible for the suppressed growth rate of cells in the non-CO₂ incubated cultures during the extended growth rate. However during this timeframe of possible limited aeration, the specific productivity of the cells had not yet decreased. It may therefore be proposed that the decrease in specific productivities initially noted was not due to a limitation in the level of aeration provided by the shake flask model. It is however possible that during the stationary phase (i.e. the period of time at which cells are at their maximum concentration), a limit in aeration may have eventually come into play, as the specific productivity of the cells did decrease again for cells cultured in both CO₂ and non-CO₂ environment over the stationary growth period. It is important to point out that assumptions being made in relation to limited oxygen availability are based on the limitations of the shake flask platform to provide adequate air supply for high cell densities cultures. It is less likely that actual diffusional limitations inside the capsules occurred for either oxygen or metabolites. Gugerli, (2003) reported that for 800 µm diameter capsules, and ~ 20 % colonisation, no diffusion limitations of metabolites or oxygen occurs.

Throughout the completion of the shake flask encapsulated cultures, the spectroscopic method for analysis of the permeability of the microcapsules to fluorescent dextrans of varying molecular weight (10-500 kDa) was optimised. The study suggested that in all 3 culture conditions, batch, control-fed perfusion non-CO₂ and control-fed perfusion CO₂, the microcapsules were permeable to dextrans of the same molecular weight of the IgG1 protein being produced by the cells, 150 kDa. Throughout the culture period the permeability of the capsules increased permitting the diffusion of the 250 kDa dextran. Towards day 9 in the control-fed perfusion cultures, some diffusion of the 500 kDa dextran was permitted, however the situation where free diffusion was possible was never reached for this dextran. The true molecular weight cut-off of the microcapsules was not determined; however an indication of the permeability of the microcapsules over time was achieved. Other authors have also investigated the permeability of APA microcapsules without determining the true molecular weight cut-off point (Wang et al, 2005; Leick et al., 2011). Ma et al., (2012) investigated the permeability of such microcapsules to fluorescently labelled BSA (67 kDa). Microcapsule permeability was correlated as the ratio of fluorescence intensity of BSA in microcapsules to that of the bulk solution. The principle behind such analysis is similar to that completed in this current work. Gugerli (2003) did however outline a method by which the actual value for the molecular weight cut-off point of APA microcapsules may be determined. In brief, the method involves incubating 1 ml of microcapsules with 1 ml of a 0.1 % (w/v) dextran polymer standard solution (ranging molecular weights 12, 50, 80, 150 and 270 kDa) in a 0.9 % NaCl w/v solution. An aliquot of liquid is withdrawn immediately from the capsule/dextran solution. A second aliquot is withdrawn following incubation with agitation for ~ 2 hours. Dextran concentrations in the solutions may be determined using HPLC analysis using an RI detector following injection onto a Shodex SB-804 HQ column. The maximal diffusion of dextran into the microcapsules corresponds to a decrease of 37 % of the initial peak height. The molecular weight cut-off of the microcapsules is defined as the lowest molar mass (M_n) of dextran for which diffusion was less than 5 % after 2 hours. In regards to cell metabolism both control-fed perfusion strategies, (non-CO₂) and (CO₂) incubated, did perform well. Glucose and glutamine were not entirely depleted at any point during the culture period. The concentration of metabolic by-products were maintained at levels consistent with that observed in the batch cultures, even though additional concentrations of glucose and glutamine were added, beyond levels observed in typical batch cultures. Some alterations in cellular metabolism did occur after perfusion was begun on day

5 in both the (non-CO₂) and (CO₂) cultures. In the non-CO₂ culture, the specific glucose consumption rate was noted to decrease by 32 % over the extended growth period (days 5-8). However during this culture period, the specific lactate production rate remained unchanged in comparison to that observed over the exponential growth period. An unfavourable shift in glucose metabolism occurred over this time point resulting in an overall yield of lactate from glucose over the entire culture period 24 % higher than that observed in the batch culture. It was stated in the results section how the concentration of glucose added in the first perfusion was over-estimated due to a 50 % decrease in the growth rate over the extended growth period in comparison to the exponential growth period. The cells were essentially over-fed, similar to that which occurred when the control-fed perfusion strategy was applied in the 1.7 L minifors reactor culture. Under conditions of high glucose concentration, it has been estimated that over 90 % of the glucose will enter glycolysis and is subsequently converted to lactate. It has been reported that, for initial glucose concentrations at or below 0.5 mM, at least half of the glucose used by rat hepatoma cells was incorporated into nucleotides; but at higher glucose concentrations 5 mM, 90 % of the glucose was converted to lactate (Miller et al., 1989). An investigation as to why the specific glucose consumption rate would decrease in this culture may suggest that the previously assumed lack of adequate aeration due to the non-vented shake flasks may have inhibited entry of glucose into the TCA cycle, which requires oxygen (Banik and Heath, 1995).

It was obvious that the some factor was limiting in the culture during this time point, verified by the reduced growth rate (-50 %) observed in this culture from days 5-8 in comparison to that observed during the exponential growth period. There was no significant difference in the specific glutamine consumption rate over the extended growth period, in comparison to that observed over the exponential growth period. This coupled with a decreased ammonia specific production rate (40 %) over the extended growth period signifies a shift to more efficient glutamine metabolism during this time period. The overall yield of ammonia on glutamine over the entire culture period was 25 % lower than that observed in batch cultures. It has been reported that at lower growth rates (which were observed over the extended growth period), significantly less ammonia may be produced per mole of glutamine consumed. (Hiller et al., 1993). This would account for the decreased specific ammonia production rate over the extended growth period in comparison to the exponential growth period.

In the (CO₂) culture the specific glucose consumption rate declined by 50 % over the extended growth period (days 5-9) in comparison to that observed during the exponential phase, this is verified by the utilisation of ~ 50 % of the glucose added during perfusion on day 5 by day 6. This was coupled with a decrease in the specific lactate production rate by 60 % over the extended growth period. The overall yield of lactate on glucose during the entire culture period was 26 % lower in the CO₂ culture in comparison to that observed in the non-CO₂ culture. A decrease in glucose conversion to lactate over time has been noted to occur for other control-fed perfusion strategies applied to mammalian cell lines. The fact that the cells were also over-fed with glucose to the same extent as the non-CO₂ culture, but the same unfavourable glucose metabolism was not noted serves to prove the point made above that the cells cultured in the non-CO₂ incubator were limited by the lack of aeration in the unvented shake flask. Miller et al., (1989) reported that it is normal for hybridoma cells to see similar shifts in lactate specific consumption as those seen in glucose consumption after addition of 8.5 mM glucose in one step. There was also a decrease in the specific glutamine consumption rate, 60 %, over the extended growth period, in comparison to that observed during the exponential growth period in the CO₂ culture. Similar to that observed in the non-CO₂ culture the specific ammonia production rate also decreased over the extended growth period. The reduction was however more profound in the CO₂ culture, as it 3.6- fold decrease in the rate was noted. Hiller et al., (1993) reported that for CHO cells cultured in suspension under perfusion conditions employing total cell retention, specific glutamine and ammonia consumption and production rates are reduced due to a shift in glutamine metabolism yielding ammonia. Goldman et al., (1998) found that for CHO cells immobilised on microcarriers and cultivated under a perfusion conditions, the presence of a direct relationship between nutrient consumption and by-product production, which was noted in the CO₂ culture, suggests a constant metabolic flux in the culture. Due to the more profound decrease in the specific ammonia production rate in the CO₂ incubated cultures, the yield of ammonia on glutamine throughout the culture period was 30 % lower than that observed in the non-CO₂ incubated cultures. For perfusion cultures using hybridoma cells, it was found that overall substrate metabolism became more efficient at high cell densities and low specific growth rates (Banik and Heath 1995). In this case, low specific growth rates were not present over the extended growth period, but higher cell densities were. Kong et al., (1998) have stated that transition to lower specific consumption and production rates does indicate

the movement to a stable perfusion environment with efficient nutrient metabolism. This was for anchorage dependent CHO cell line on microcarriers, no bleed.

As noted above there were alterations in the growth, metabolic and production profiles for the encapsulated CHO DP-12 cells cultured under batch and control-fed perfusion conditions. Differences were noted in the same cellular activities between the control-fed perfusion cultures carried out in both the non-CO₂ and CO₂ incubators. All of such parameters had the possibility to alter the quality of the IgG1 protein in terms of the profile of N-linked glycan structures attached and also the aggregated state of the protein.

It was noted above how there was a slight increase in complexity of the glycoform profile over the stationary growth period in the batch encapsulated culture, attributed to the lack of depletion of glucose over this growth phase, which prevents a depletion of nucleotide sugar donors UDP-GlcNAc at the intracellular level (Nyberg et al., 1999). The application of the control-fed perfusion strategy to a culture under comparable conditions (non-CO₂ incubated) to the batch culture on day 5 resulted in increases and decreases in the relative % of glycans present on the IgG1 protein. Such increases and decreases were noted to occur primarily over the extended growth period (days 5-8), in which the cells continued to grow, but at a 50 % lower rate than that observed over the exponential growth period (days 1-5). The majority of the changes which occurred in the batch culture did so over the stationary growth period. In the control-fed batch culture only two glycans increased in relative % of the stationary phase, including M5 and F(6)A2G2. Some of the changes which did occur in the control-fed batch culture over time were more profound than those which occurred in the batch culture. The relative % of each type of glycan present on the IgG1 was compared and contrasted at the end of the stationary phase in the batch and control-fed perfusion cultures. It was found that the relative % of M5 was 2-fold higher than that observed in the batch. The complex glycan F(6)A2G2 was 30 % higher in the control-fed culture than the batch and the non-complex glycan F(6)A2 was 13 % higher in the batch culture than the control-fed perfusion culture. Therefore regardless of M5 increases, a further degree in complexity of the glycans was found in the control-fed perfusion culture in comparison to the batch. This can be associated with the prevented depletion of nutrients, glucose and glutamine, which are key precursors for nucleotide sugar donors involved in glycan chain extension (Nyberg et al., 1999). As reported in the study so far, M5 appears to be the glycan which increases over time in all encapsulated culture conditions tested. So far in this thesis, increases in M5 have been noted to occur to the greater extent when glucose or glutamine have been limiting. This was the

proposed explanation made above for the higher relative % of M5 present in 1.7 L batch encapsulated cultures, in comparison to that observed in the 1 L Erlenmeyer batch encapsulated shake flask cultures. However a lack of metabolites is not a possible explanation for the higher relative % of M5 present in the control-fed perfusion culture in comparison to the batch culture. A possible cause for the notably higher levels of this glycan in the control-fed perfusion cultures at the end of the stationary phase in comparison to the batch cultures (1 L shake flask and 1.7 L minifors reactor platforms) may be associated with an increase in osmolality in the culture environment brought about by the addition of fresh culture media. For a control-fed perfusion culture, Teng et al., (2011) reported an increase in osmotic pressure over the course of the culture period. Pacis et al., (2011), investigated how increasing osmotic pressures in a cell culture environment could result in increased mannose levels on recombinant IgG proteins. They reported an increase in M5 levels for protein harvested from cultures with increasing osmolalities. IgG from 4 cell lines which typically has low levels of M5 were tested under increased osmolality conditions. The overall result was an increase in M5 as osmolality was increased. Overall though in regards to protein quality it must be stressed that the levels of M5 present would be considered low levels for all culture conditions tested (batch or control-fed perfusion). It is however important to note that a prolonged control-fed perfusion culture could result in higher levels of this glycan. Pacis et al., 2011 highlighted that the main concern regarding high mannose level, in that the bioactivity of a therapeutic antibody may be adversely affected.

For the majority of cases in which the effect of a perfusion strategy on recombinant protein quality was investigated, an investigation of the effect the strategy may have on protein integrity and function was performed. Ryll et al., (2000) has reported on the effectiveness of a perfusion strategy in reducing proteolysis and maintaining protein integrity throughout the process. A similar report was made by Link et al., (2004). Werner et al., (2002) reported on the maintenance of protein functionality when changing from a batch to a perfusion mode of production. In regards to the impact of perfusion culture modes on protein glycosylation, Wang et al., (2002) reported no significant differences in the glycosylation pattern present on recombinant proteins in comparison to proteins harvested from batch culture conditions for CHO cells immobilised on microcarriers producing erythropoietin (EPO). Alternatively for an IFN- γ producing CHO cell line immobilised on microcarriers, a more complex glycosylation profile was achieved under a perfusion mode of cultivation in comparison to a batch mode (Goldman et al., 1998). They reported on a higher level of doubly galactosylated

glycans, similar to the findings in this current study, and increases sialylation in the perfusion culture in comparison to the batch culture.

The quality of IgG harvested from the 5 % CO₂ incubated culture was compared and contrasted to that harvested from the non-CO₂ incubated culture. As noted above there were alterations in cellular behaviour noted between the CO₂ and non-CO₂ incubated control-fed perfusion cultures. They were notably a higher growth rate (~50 %) in the CO₂ culture over the extended growth period (days 5-9), a lower specific production rate over the extended growth period, 43 %, and over the stationary growth period, of 16 %. Cellular metabolism was also deemed to be more favourable in the 5 % CO₂ incubated culture in comparison to the non-CO₂ incubated culture due to the lower yields of waste products on metabolites noted. It has been extensively reported how increased cellular growth rates may have negative effects on protein glycosylation, as it reduces the time in which the recombinant protein is exposed to the glycosyl transferase enzymes present in the endoplasmic reticulum (ER) and golgi apparatus (GA), as review by (Butler, 2006). Alternatively reduced specific protein production rates are thought to be beneficially in increasing the time the protein spends in the ER and GA compartments exposed to the glycosyl transferase enzymes (Hooker et al., 1999). Efficient glucose and glutamine metabolism is associated with overall good cellular metabolic performance allowing for such metabolites to be utilised for the production of nucleotide sugar precursors (Nyberg et al., 1999). The glycosylation of the IgG1 protein harvested from both the control-fed perfusion cultures was compared and contrasted at time points after the extended growth period, (day 8 for the non-CO₂ incubated culture and day 9 for the CO₂ incubated culture). Protein samples were also harvested at the end of the stationary growth phases in both cultures for comparison of protein quality, (day 11 for non-CO₂ incubated culture and day 13 for CO₂ incubated culture). By the end of the extended growth period, the glycoform profile of IgG1 from both cultures was almost identical. This indicates that the alterations in growth rate, specific production rate and metabolic profiles did not impact on the quality of the protein harvested from both culture conditions investigated. The main exception was that the relative % of F(6)A2G2 which only increased by 59 % over the extended growth period in the CO₂ culture, in comparison to the 79 % increase observed in the non-CO₂ culture. This greater increase did not however result in a significant difference being present in the relative % of this glycan between the two culture conditions at the end of the extended growth period.

It may therefore appear that the 5 % CO₂ control-fed perfusion culture was more beneficial in achieving higher cell numbers, higher volumetric productivity without impacting on the quality of a recombinant protein. However a comparison of glycosylation profile of the IgG1 harvested from the non-CO₂ and 5 % CO₂ cultures at the end of the stationary phase did indicate that the quality of the protein was compromised over time in the CO₂ culture. As noted above only significantly low changes in the relative % of two glycans, M5 (+15 %) and F(6)A2G2 (+ 11 %), occurred over the stationary growth phase in the non-CO₂ culture. It is common to see an increase in M5 over time. This is not considered detrimental to protein quality unless a subsequent decrease in the relative % of a complex glycan also occurs, which did not happen in this case. For the 5 % CO₂ culture extreme increases in non-complex glycans (F(6)A1/A2, F6A2 and M5) were reported and were coincided with extreme decreases in complex types glycans (F(6)A2[6]G(4)1, F(6)A2[3]G(4)1, F(6)A2G2, A2G2 and F(6)A2G2S1). Such alterations signify the action of glycosidase enzymes which are released from cells brought about by cellular apoptosis. Goldman et al., (1998) reported a concomitant reduction in the sialic acid content of IFN- γ during the death phase. Wong et al., (2005) found that when culture viability decreased, the sialic acid content of the produced recombinant protein IFN- γ also decreased. It is also important to note that no pH control was utilised in these shake flask cultures. Sialidase activity is likely to increase as the pH decreases (Gramer and Goochee (1993)). However the same study did also report sialidase activity at optimal culture pH around 7. As the culture was conducted in a CO₂ environment, the culture may have become more acidic than the non-CO₂ culture resulting in enhanced sialidase activity. The viability of the cells was lower in the perfusion CO₂ culture than that observed in the perfusion (non-CO₂), but did still remain above 63 % on day 13. However it is important to note that as the total cell number was higher in the control-fed perfusion CO₂ culture, than the non-CO₂ culture, the total number of dead cells releasing glycosidase enzymes would have been higher than present in the non-CO₂ culture, even if the % viability was not dramatically lower in the CO₂ culture. Other authors have reported on the effectiveness of a perfusion strategy for the removal of proteases and glycosidase which impact on protein quality (Ryll et al., 2000). In the case of the control-fed perfusion CO₂ culture, the perfusion rate was increased to two medium changes per day after day 9. However this increased rate was not sufficient to alleviate the activity of the glycosidase enzymes. It is well known that when a perfusion culture is operated with no bleed, cell viability does become a limiting factor in the process as under steady state conditions the cell

death rate must equal the cell growth rate eventually. When determining the ideal bleed rate for a given perfusion process it should first be established whether viability is an important parameter with respect to product quality, otherwise the bleed rate should be chosen as low as possible. If low viabilities are should be avoided the bleed rate should be chosen higher, depending on the viability desired. To obtain the highest volumetric productivity the feed rate should be set at such a rate that the cells are just in the non-nutrient limited range (Chon and Zarbis-Papastoitsis, 2011).

6.4: Conclusion

The final chapter of the thesis did allow for the overall aim of the project to be met which is “High cell density CHO cultures: improved productivity and product quality”. The application of the control-fed perfusion strategy to encapsulated shake flask cultures performed in a 5 % CO₂ incubator has led to ~5–fold improvements in comparison to batch encapsulated shake flask cultures and <10- fold improvements in comparison to batch suspension cultures in the maximum viable cell density achieved (cells/ml_{shakeflask}). Increased productivity has been brought about in regards to volumetric concentrations reached. The control-fed perfusion culture performed in the 5 % CO₂ incubator brought about ~2.6 fold increases in total IgG1 produced in comparison to that achieved for batch encapsulated cultures. Product quality was also improved denoted by the increases in relative % of complex glycans which occurred in the two control-fed perfusion cultures over the extended growth period, in comparison to that in the batch cultures for the entire culture period. In the achievement of such high quality protein titres, the aggregation of the protein was not noted to be affected.

The work has highlighted the requirement for stricter control of cell viability in encapsulated perfusion cultures. Due to the lack of dead cell removal from the cultures, protein quality did become compromised over the stationary phase in the high density control-fed perfusion cultures performed in the 5 % CO₂ incubator. Under conditions of full cell retention, using microcapsules, complex glycan structures present on recombinant proteins are subjected to glycosidase cleavage. This study has therefore outlined a limitation in the application of microcapsules as retention devices for perfusion cultures. Although the viability of the culture was noted not be particularly low (~63 %), the fact that the culture was a high density culture did result in a higher number of total dead cells than would have been noted over the stationary phase in any of the other culture conditions tested.

Possible solutions to the aforementioned problem would include introducing a cell bleed by removing capsules with spent media from the culture. This would serve to reduce the number of dead cells in the culture environment. However this would also compromise the high cell density/ml_{vessel} achieved in these cultures. Another possible step forward could include lowering the seeding density by reducing the capsule inoculum volume. Such efforts may lead to enhanced capsule colonisation and also reduce the cells/ml_{reactor}. The number of dead cells would be reduced due to the decreased cells/ml_{reactor} in the shake flask environment.

A much more attractive step forward would be to move the control-fed perfusion cultures back to the minifors reactor platform. Such platforms allow for tighter control and monitoring of cell numbers, viabilities and nutrient requirements as the platform facilitate the use of various Process Analytical Technologies (PAT) tools which enable online, continuous monitoring of cell growth and nutrient requirements. Should cell viabilities not however be enhanced through stricter control and monitoring, a second benefit of the reactor platform is in the feasibility it provides for the continuous addition and removal of fresh and spent media respectively. The residence time in which the protein (present in spent media) spend in the culture environment is thus reduced, reducing the overall time the protein is exposed to glycosidase enzymes. The minifors reactor platform model is also capable of supporting higher cell densities than the shake flask model due to better aeration and agitation. Shake flasks models are associated with low cell titre applications ($2-4 \times 10^6$ cells/ml). In this study we have pushed the boundaries of the shake platform for cell numbers attainable. Moving back to the minifors reactor platform would also alleviate any diffusional limitations associated with high density shake flask cultures which could have 2 benefits. There is the potential to enhance cell viability. Also a second limitation was noted in both of the control-fed perfusion shake flask cultures which were the reduced specific production rate over the stationary phases attributed to the incapability's of the shake flask design to support such high cell densities.

A movement back to a minifors reactor platform does however require one major limitation to be overcome and that is the stability of the APA microcapsules. It is only in recent publications that efforts have been noted to be made for enhanced stability of APA microcapsules, and which have been discussed in this Chapter. Possibilities for enhancement in microcapsule strength are associated with increased poly-L-lysine concentrations, number of layers and also different forms of poly-L-lysine have been investigated. The quality and viscosity of alginate used also has potential for effecting APA microcapsule strength.

Microcapsule size has been noted to potential influence the mechanical resistance of APA microcapsules.

Once such stability limitations have been overcome, this current work illustrates the potential for encapsulation as a cultivation technique to achieve high cell densities (+ 10- fold) and higher volumetric productivities in comparison to suspension. Mainly this work has demonstrated the potential for enhanced glycan complexity in such cultures once tighter control of cell viability, or increased perfusion rates have been achieved.

Chapter 7: Overall Thesis Conclusions and Outlooks

Abbreviations

CHO	Chinese hamster ovary
CPP	critical process parameter
CQA	critical quality attribute
FDA	food and drug authority
L-GLN	L-glutamine
IgG1	Immunoglobulin 1
IR	Infrared spectroscopy
mAb	monoclonal antibody
PAT	Process analytical technology
STR	stirred tank reactor

A recent review by Butler and Meneses-Acosta (2012), outlined that the specific productivity of recombinant glycoproteins from Chinese hamster ovary (CHO) cell lines can be expected to be above 50 pg/cell/day giving rise to culture systems with titres of around 5 g/L with the application of suitable culture platforms (e.g. fed-batch platforms). This is a substantial increase in comparison to 10 pg/cell/day noted from such cell lines a decade ago. Improvements in bioprocessing have seen cell numbers in a typical culture rise from an estimated 1 million cells/ml over a 7 day culture period to 10 million cells/ml with culture periods being extended to 21 days for enhanced volumetric productivity. The biopharmaceutical sector competes well with all other sectors in the pharmaceutical industry with reported sales of biologics reported at US\$120 billion for 2011 and expected to reach \$150 billion by 2015. Global sales of monoclonal antibodies (mAbs) alone are expected to reach US\$58 billion by 2016.

Such expected increases in the bioprocessing industry result in a persistent demand for the optimisation of upstream mammalian cell culture processes, with the aim of identifying critical process parameters (CPP'-s) affecting cell growth and productivity and subsequently developing effective tools to be put in place for both the monitoring and control of these CPP'-s. The process analytical technology (PAT) framework initiative introduced by the food and drugs authority (FDA) in 2001, outlines requirements for the identification of CPP'-s and subsequent monitoring and controlling enabling the production of recombinant proteins of

defined and consistent qualities. The critical quality attributes (CQA) determining the quality of a protein include both the glycosylated and aggregated states of the protein. Taking into account the constant need for increased cell and product yields, while keeping in mind the impact of alterations in process conditions on protein quality, the project entitled “High density CHO cell cultures: improved productivity and product quality” was designed.

The project set about to firstly identify possible growth limiting substrates for the in-house CHP DP-12 cell line producing the recombinant protein Immunoglobulin 1 (IgG1) (Chapter 3). The substrate of interest for the study was the amino acid L-glutamine (L-GLN). This amino acid and its by-product ammonia are considered CPP's affecting cell growth, productivity and outwardly product quality. The inefficient energy metabolism of mammalian cells in culture is something which is known to be limiting the optimisation of upstream processes. A dynamic study was therefore designed allowing for the growth, metabolic activity, productivity and product quality over a range of L-GLN concentrations (0-12 mM) in small scale, 1 L Erlenmeyer shake flask studies to be determined. This initial work successfully identified the requirement for L-GLN in mammalian cell cultures. The absence of L-GLN resulted in a decreased cell yield and specific productivity rate of 45 % and 2.2-fold respectively in comparison to cultures containing L-GLN. The quality of the protein was compromised by a lack of L-GLN characterised noted by the occurrence of significantly higher levels of the non-complex high mannose glycan M5 and lower level of the complex galactosylated and sialylated glycan F(6)A2G2S1. The study identified that at a concentration of 4 mM L-GLN, exponential cell growth ended with the depletion of this nutrient. Although an increased initial L-GLN concentration of 12 mM did not enhance the culture longevity or maximum cell yields beyond that achieved in the 4 mM L-GLN culture, this study did demonstrate that the increased yield of ammonia (13 mM) over the culture period in the 12 mM L-GLN culture did not have a negative effect on cell growth, productivity or product quality in comparison to the results observed for the 4 mM L-GLN culture (in which the yield of ammonia was ~5 mM). These results were important as at later stages in the project it was predicted that if higher cell densities were to be achieved, than those noted in the 4 mM L-GLN culture, the concentration of L-GLN in the media may have to be increased in order to meet the specific requirements of a higher cell density. It may be argued that this work is not novel due to the vast amount of publications already acknowledged focusing on the effect of ammonia on the glycosylation or recombinant proteins. However in starting a project which would lead onto more novel work, i.e. identifying CPP's in high density encapsulated

cultures affecting product quality, it was firstly necessary to identify our window for optimisation of cell growth and productivity through the manipulation of culture components. The vast array of literature on this subject concerning the effects of L-GLN and ammonia on cell behaviour does state that all of the effects of L-GLN and ammonia can be found to be cell line specific. Although the work was not novel in its own right, an interesting observation was made in the dynamic study which was conducted. In previous reports focusing on ammonia as a critical culture component effecting cellular activities and product quality, experiments were conducted by spiking cultures with high initial ammonia concentrations. A comparison made in this study between cellular activities in cultures in which high levels of ammonia accumulates and high levels of ammonia are initially present (5-15 mM), found an over-estimation of the negative impact of ammonia on all cellular activities in cultures containing high initial ammonia concentrations in what was previously reported. For example there was a 50 % lower level of sialyated glycans present on protein harvested from the 15 mM ammonia culture, in comparison to the 12 mM L-GLN culture. The study also identified that should ammonia later become a problem in high cell density cultures, switching to less ammoniagenic substrates, such as glutamate would hamper cell growth (3.25- fold decrease), product quality (increased accumulation of non-complex glycans) in comparison to cells cultured in the presence of L-GLN.

Having completed these initial studies at small scale it was then necessary to up-scale cultures to a 1.7 L minifors reactor platform (Chapter 4). The previous studies were completed at small scale due to the ease at which efficient identification of effects of L-GLN and ammonia could be conducted and the feasibility of performing such a dynamic study at small scale platform. Upscaling was completed due to a general understanding that stirred tank reactor platforms allow for better aeration, agitation and also that shake flasks are associated with low yields and cell titres. The upscaling of 4 mM L-GLN cultures from shake flasks to minifors reactors had a negative effect in the movement towards achieving higher cell densities and productivities. The maximum cell number achieved and specific production rate displayed by the cells were both 27 % lower than that observed in shake flasks. In 4 mM L-GLN shake flasks cultures, a cessation in cell growth was noted to occur when the L-GLN was depleted. However in minifors reactor experiments there was a shift in cellular metabolism with glucose being depleted earlier on in the culture in an unfavourable metabolism which saw increased yields of lactate. Product quality was subsequently negatively affected with a 32 % decrease in the level of complex double galactosylated

glycans from protein harvested from 1.7 L minifors reactor cultures in comparison to shake flask cultures. It was identified that the cells were stressed in some way in this environment in comparison to the shake flask environment which does not incorporate such high levels of shearing mechanical agitation and aeration in comparison to a stirred tank bioreactor.

In moving forward to achieve high density cell cultures and identify impacts of productivity and product quality, there were two options from this study: (I) optimise conditions in reactor through manipulation of process parameters such as agitation and aeration. However prior research surrounding the topic suggested that the results of previous investigations had limited success. Therefore a second option, (II), encapsulation of the cells in a gelled core surrounded by a shear protecting matrix of alginate-poly-L-lysine (APA), seemed to demonstrate much more potential. The theory of immobilisation/entrapment/encapsulation of cells inside of a gelled matrix was first developed with the aim of offering protection to cells, Encapsulation of the CHO DP-12 cell line in APA microcapsules would potentially prove beneficial as a step forward from Chapter 4 due to the it's shear protecting potentials. There is also a second benefit of cell encapsulation which adheres to the overall title of the thesis of high cell density. The microcapsules environment provides a 3-D structure for the growth of cells in close proximity enhancing cell to cell communication and increased local concentrations of growth and productivity promoting autocrine factors. Almost all publications focusing on cell encapsulation or immobilisation are coupled with the phrase "High cell density" referring to a situation in which higher cell densities for cell lines may be achieved, greater than that which occurs in suspension. For the particular CHO DP-12 cell line used in this study encapsulation brought about 3.7- and 4.8- fold increases in cell and product yield in comparison to cells cultured in suspension in the minifors reactor platform. Although encapsulation of cells for enhanced growth and productivity has been reported previously for various mammalian cell lines, the novelty in this chapter came in the identification of the effect of this culture method on protein quality. With regards to the identification of important parameters for monitoring and controlling, altered metabolic profiles for glucose and glutamine were noted between the suspension and encapsulated cells. With regards to product quality, a more profound increase in the level of non-complex glycan M5 was noted to occur over the stationary phase in encapsulated cultures in comparison to suspension cultures. This was attributed to the increased stationary phase in encapsulated cultures and subsequently the increased time frame over which cells were producing protein

in a state of L-GLN depletion. The depletion of L-GLN as an issue was attributed to the batch nature of the culture.

An step forward from the encapsulated cultures was to design a feeding strategy which would eliminate the problems associated with nutrient depletion in batch cultures and facilitate the enhanced colonisation of the microcapsule above the level reported in the batch cultures (5 %) (Chapter 6). After an analysis of various feeding strategies, a control-fed perfusion strategy was designed mainly due to the benefits associated with perfusion in that the residence time of the protein in the reactor was reduced. Also looking forward to potential monitoring and control of cultures, a perfusion strategy would allow for the ease of control of nutrient and by-product concentrations through the addition and removal of media. Successful efforts were made using a control-fed perfusion strategy and colonisation was enhanced to 11 % and the degree of M5 was reported to be lower at the end of the control-fed perfusion culture than that reported for the batch culture in 1.7 L minifors reactor platforms.

It was however also at this stage in the project that the first limitation was met, namely the instability of the microcapsules as cell retention devices. Loss of capsules integrity due to break up was noted to a degree of 58 % between days 7 and 9 in the control-fed perfusion cultures. Steps forward from the first study in Chapter 6 did not focus on making stronger capsules. The overall aim of the thesis was to promote high cell densities and to investigate the implications on productivity and product quality. Therefore a decision was made to take the encapsulated experiments back to the shake flask model, in which the characteristically weak capsules could possibly withstand reduced shear forces in a shake flask platform. The overall aim of the thesis was met with dramatic increases in cell and product yields through application of the control-fed perfusion strategy to encapsulated cells in shake flasks. The degree of colonisation in the final control-fed perfusion encapsulated study in the CO₂ incubator resulted in a degree of colonisation of 27 %. Table 7.1 below details the increased cell yields achieved over time scales of the experiment. Although a decrease in specific productivity was reported in control-fed shake flasks cultures, particularly over the stationary growth phase, increased volumetric product yields did increase over the timescale of the experiments conducted.

Table 7.1: Overview of enhancements in maximum viable cell number attained and degree of capsule colonisation over the entire timescale of the project.

Timescale	Culture	Culture mode	Max VCD (*10⁶ cells/ml)	% Capsule colonisation
Chapter 3	1 L Erlenmeyer shake flask (suspension)	Batch	4.30 +/- 0.19	
Chapter 4	1.7 L minifors reactor (suspension)	Batch	3.12 +/- 0.27	
Chapter 5	1.7 L minifors reactor (encapsulated)	Batch	11 +/- 1.35	5
Chapter 6	1 L Erlenmeyer shake flask (encapsulated)	Control-fed perfusion (non-CO ₂)	18.5 +/- 1.33	11
Chapter 6	1 L Erlenmeyer shake flask (encapsulated)	Control-fed perfusion (CO ₂)	44.9 +/- 2.54	22.7

The control-fed perfusion strategy was also noted to be beneficial in regards to protein quality as increases which occurred in complex glycans throughout the extended growth period (days 5-8/9) in the control-fed perfusion cultures, were greater than those which occurred in the batch cultures when encapsulated cultures were conducted in shake flasks. This is attributed to the constant supply of nutrients for the synthesis of nucleotide sugar precursors for the supply of glycan sugars. The control-fed perfusion cultures in shake flasks did not reduce the level of the high mannose non-complex M5 glycan in comparison to levels observed in the batch cultures. However it was pointed out that the relative % of M5 in regards to other glycans present on the protein was considered relatively low. It is something that would have to be monitored should the process time be extended through further optimisation due to the tendency to increase over time. Therefore it may be concluded that irrespective of M5, the control-fed perfusion encapsulated cultures did result in a higher level of glycan complexity by the end of the extended growth period in comparison to the batch cultures.

It was however in the stationary growth period (day 9 onwards) in the control-fed perfusion culture in the CO₂ incubator that complexity of the glycans were adversely effected by apparent glycosidase activity resulting in the increase in the relative % of non-complex glycans coupled with a similar decrease in complex glycans. A second limitation in the study was thereby identified. This was attributed to the ability of the capsules to allow for complete cell retention. In continuous cultures, the bleeding of cultures is beneficial in removing dead

cells from the reactor whose released enzymes pose a threat to product integrity and quality. Microcapsules as retention devices do not permit the bleeding of cells from a perfusion system. Even though the viability of the cells was similar to that of the control-fed perfusion culture conducted in the non-CO₂ incubator during the stationary phase, the total (live + dead) cell number was higher in the CO₂ culture and so there was a higher number of dead cells releasing glycosidase enzymes. A recommendation could be that that total number of cells in the culture be reduced by engaging in cell bleed through means of removing some capsules in spent media. An ideal time to engage in this activity would be at the beginning of the stationary phase. The number of dead cells present in the culture would be reduced over the stationary phase, which was a critical time point for the protein quality. This would however reduce the beneficial effect of increased cell density (cells/ml_{reactor}) which was noted in this control-fed perfusion CO₂ culture. However it was noted in this culture that the specific productivity did during the stationary growth phase and that both productivity and product quality are more favourable over the extended growth period before stationary phase is reached.

A more attractive step forward however would be to return the control-fed perfusion encapsulated cultures to a stirred tank reactor (STR) platform. Such platforms enable the application of elaborative, online and continuous controlling and monitoring PAT tools which specialise in both cell growth and viability monitoring, through dielectric spectroscopy, and also nutrient concentration monitoring, through the application of Infrared spectroscopy (IR). As this project was completed in a shake flask, online monitoring and control of cell growth and viability was not possible. This project has identified the need for online monitoring of cell viability through PAT technology so as that product harvest time or capsule removal (dead cell bleeding) may be optimised in favour of product quality. The cells in the control-fed perfusion cultures displayed the typical growth and death cycle characteristics displayed by mammalian cells in a fully retained perfusion environment. The use of the PAT technologies in a controlled larger scale STR environment would enable stricter control of the perfusion strategy and also the application of a strategy with continuous removal and addition of media, in which the concentration of nutrients may be monitored online continuously to meet the cellular demands. The result of such an application may extend the time period over which the quality of the protein is not affected by cell viability, as further metabolic optimisation of the cells would be enabled. It is also important to point out that the continual removal of spent media, which an STR facilitates, reduces the residence time of the protein in

the bioreactor environment. Therefore if similar losses in cell viability did occur, even with the application of stricter metabolic control, the timeframe for which the protein would be exposed to glycosidases would be dramatically reduced with continuous spent medium removal. An STR environment would also serve to alleviate any diffusional limitations which are associated with the shake flask environment, traditional used for low volumetric and low cell titre applications ($2-4 * 10^6$ cells/ml). This project has essentially pushed the boundaries of the shake flask model (cell densities achieved $\sim 4.5 * 10^7$ cells/ml_{reactor}) and generated some promising results in regards to the possibility of not only increased cell and product yields but also higher product quality by means of encapsulation and designed feeding.

Before going back to an STR model, the initial limitation in this project concerning capsule instability must first be addressed. There is great potential to increase capsule stability and this work has provided the basis for a new PhD project currently being conducted with the overall aim to identify possible mechanisms to either (i) enhance the stability of the traditional APA microcapsule and/or (ii) also to develop new microcapsules using polymers that have been noted for enhanced stability and biocompatibility for mammalian cell culture. Possible mechanisms to enhance the stability of the APA microcapsules have been outlined in Chapter 6 and include:

- Smaller sized capsules: for enhanced stability (Zhang et al., 2007)
- Movement from liquid core to gelled core: Brequet et al., (2007) reported on the increased strength associated with semi-liquid core alginate microcapsules. Such microcapsules may be formed through the elimination of the incubation in 0.05 M sodium citrate step. Other authors have also reported on the enhanced stability of solid core microcapsules in comparison to liquid core. Both solid and semi-liquid core microcapsules were eliminated from this current study which aimed to investigate high cell density. Increased colonisation may have been inhibited in these capsules due to space limitation due to the presence of solid alginate in the core.
- Increasing the concentration of poly-L-lysine and incubation time: Reports have suggested an increased in concentration of poly-L-lysine increases capsule stability (Leick et al., 2011) or incubation time could enhance capsule strength and subsequent integrity. Brequet et al., (2007) found that smaller capsules lost resistance faster, however this was attributed to the fact that they were incubated for shorter time periods than larger ones in poly-L-lysine.

- Increasing the number of layers of poly-L-lysine surrounding the bead: Brequet et al., (2007).
- Different types of poly-L-lysine including poly-D-lysine, poly-L-ornithine (Castro et al., 2005) and poly-ε-lysine (Ma et al., 2012)
- Increasing the viscosity of alginate in the 0.03 % outer layer
- Inclusion of calcium ions in cell culture media
- Additional polymers (Wang et al., 2005)

It is important to point out that at no point during this project was protein aggregation considered an issue. This is attributed to the fact that the APA microcapsules noted to permit the diffusion of the IgG1 protein. Further development of microcapsules in an effort to enhance stability could have an effect on the molecular weight cut-off of the microcapsule to a recombinant protein of interest and result in high concentrations of protein inside microcapsules leading to concentration induced protein aggregation. Therefore as part of microcapsule stability optimisation it is important that there is no trade off in the non-aggregated production of a rIgG protein.

The majority of the references listed above are within the last 5 years and therefore suggest that the application of APA microcapsules is becoming increasingly attractive, aside from the benefits observed in this project. The results of this project are of commercial interest. It was stated above that industrial cell lines are expected to grow in the range of $10 * 10^6$ cells/ml. This project has demonstrated the possibility to achieve an ~ 11- fold increase in cell number for a cell line which typically grew to $4 * 10^6$ cells/ml_{vessel}. It is therefore feasible to suggest that the application of the methods used in this project to industrial cell lines could in turn result in cell concentrations upwards of $110 * 10^6$ cells/ml. The fact that such achievements were made without the application of novel and advanced control and monitoring tools really does demonstrate the advantages of the cultivation methods investigated in this project.

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Appendices

Appendix A: Calculation of maximum shear stress exerted on cells cultured in 1.7 L minifors reactor (Chapter 4)

Table A1: Values for all parameters of the 1.7 L minifors reactor, required for the calculation of maximum shear stress on cells cultured in the reactor.

Parameter	Value
Working volume (V_L)	1.2 L
Vessel diameter (D_T)	10.5 cm
Air flowrate	0.06 L/min
Impeller diameter (D_i)	6.7 cm
Impeller thickness (T_i)	0.13 mm
Impeller width (W_i)	6.9 cm
Agitation rate (N)	100 rpm
Culture medium density (ρ)	1.005 g/ml
Culture medium kinematic viscosity (ν)	0.0092 cm ² /s

Theoretical values of maximum shear stress under stirred conditions, τ_{\max} (dyn/cm²), can be determined through the evaluation of Equation (A1).

$$\tau_{\max} = 5.33 \cdot \rho \cdot (\varepsilon_t \cdot \nu)^{1/2} \quad (\text{A1})$$

where ρ denotes fluid density and ν denotes the kinematic viscosity. ε_t is the total energy dissipated per unit mass and in sparged conditions is defined by equation (2.2.2.1.2) as described by Fernandes-Platzgummer et al., (2014).

$$\varepsilon_t = \varepsilon_{is} + \varepsilon_s \quad (\text{A2})$$

ε_s is the energy dissipation rate from the sparging given by:

$$\varepsilon_s = V_s \cdot g \quad (\text{A3})$$

where v_s denotes the superficial gas velocity (cm/s) and g is the gravitational constant (981 cm/s²). v_s is determined by the following equation:

$$v_s = \frac{4Q_{air}}{\pi D_i^2} \quad (A4)$$

where Q_{air} is the air flow rate (cm³/min) and D_i in the impeller diameter.

For the minifors reactor experiments: $Q_{air} = 0.06$ L/min, which is $6 * 10^{-5}$ m³/min

1 m³/min = 1 000 000 cm³/min, therefore $6 * 10^{-5}$ m³/min = 60 cm³/min

$$v_s = \frac{4(60\text{cm}^3 / \text{min})}{\pi(6.7\text{cm})^2} = \frac{4(1\text{cm}^3 / \text{s})}{\pi(44.89\text{cm}^2)} = 0.0284 \text{ cm/s}$$

From above $\varepsilon_s = v_s \cdot g$ therefore $\varepsilon_s = (0.0284\text{cm} / \text{s}) * (981\text{cm} / \text{s}^2) = 24.32 \text{ cm}^2/\text{s}^3$

ε_s is often described as W/kg which is the same as m²/s³.

1 W/kg = 1 m²/s³ so therefore we must convert 24.32 cm²/s³ to m²/s³.

24.32 cm²/s³ = 0.0024 m²/s³ or 0.0024 W/kg

$$\underline{\varepsilon_s = 24.32 \text{ cm}^2/\text{s}^3 \text{ or } 0.0024 \text{ W/kg}}$$

ε_{is} is the energy dissipation rate from the impeller under sparged conditions given by:

$$\varepsilon_{is} = \frac{P_s}{V_L \rho} \quad (A5)$$

where V_L is the working volume of the reactor and ρ is the fluid density. P_s is the power consumption under sparged conditions and may be determined from the following equation:

$$P_s = Np_s N^3 D_i^5 \rho \quad (A6)$$

where N is the agitation rate (s⁻¹). Np_s is the dimensionless power number under sparged conditions. As described by Fernandes-Platzgummer et al., (2014), $Np_s \approx Np$ for air flowrates typically used in mammalian cell culture. The power number Np can be calculated

from Nagata correlations (Sen et al., 2002), as described by Fernandes-Platzgummer et al., (2011).

$$Np = \frac{K_1}{Re} + K_2 \cdot \left[\frac{(10^3 + 1.2 \cdot Re^{0.66})}{10^3 + 3.2 \cdot Re^{0.66}} \right]^{K_4} \quad (A7)$$

where,

$$K_1 = 14 + \left(\frac{W_i}{D_t} \right) \cdot \left[670 \cdot \left(\frac{D_i}{D_t} - 0.6 \right)^2 + 185 \right] \quad (A8)$$

where,

$$K_2 = 10^{K_3} \quad (A9)$$

$$K_3 = 1.3 - 4 \cdot \left(\frac{W_i}{D_t} - 0.5 \right)^2 - 1.14 \cdot \left(\frac{D_i}{D_t} \right) \quad (A10)$$

$$K_4 = 1.1 + 4 \cdot \frac{W_i}{D_t} - 2.5 \cdot \left(\frac{D_i}{D_t} - 0.5 \right)^2 - 7 \cdot \left(\frac{W_i}{D_t} \right)^4 \quad (A11)$$

where W_i is the impeller width, D_t is the vessel diameter and Re is the Reynolds number.

$$Re = \frac{D_t^2 \cdot N}{\nu} \quad (A12)$$

In the equation for A12, $N = 100$ rpm

$$100 \text{ rpm} = 10.47 \text{ rads/s}$$

We know that $1 \text{ rads/s} = 0.159155 \text{ hz}$

$$10.47 \text{ rads/s} = 1.67 \text{ hz or } 1.67 \text{ s}^{-1}$$

$$\text{Therefore } Re = \frac{(6.7 \text{ cm})^2 \cdot (1.67 \text{ s}^{-1})}{0.0092 \text{ cm}^2 / \text{s}} = 8148.51$$

N_p is subsequently determined by the evaluation of $K_1, K_2, K_3,$ and K_4 from equations A8 - A11 from above.

$$K_1 = 14 + \left(\frac{6.9}{10.5}\right) \left[670 \cdot \left(\left(\frac{6.7}{10.5}\right) - 0.6 \right)^2 + 185 \right] = 136.81$$

$$K_3 = 1.3 - 4 \cdot \left(\frac{6.9}{10.5} - 0.5\right)^2 - 1.14 \left(\frac{6.7}{10.5}\right) = 0.4738$$

$$K_2 = 10^{0.4738} = 2.9771$$

$$K_4 = 1.1 + 4 \cdot \left(\frac{6.9}{10.5}\right) - 2.5 \cdot \left(\frac{6.7}{10.5} - 0.5\right)^2 - 7 \left(\frac{6.9}{10.5}\right)^4 = 2.3755$$

$$N_p = \frac{136.81}{8148.51} + 2.9771 \left[\frac{(10^3 + 1.2(8148.51)^{0.66})}{10^3 + 3.2(8148.51)^{0.66}} \right]^{2.3755} = 1.11$$

From Equation A6:

$$P_s = (1.11) \cdot (1.67s^{-1})^3 \cdot (6.7cm)^5 \cdot (1.005g/ml) = 70147 (cm^5) \cdot (s^{-3}) \cdot (g/ml)$$

$$\text{Therefore } \varepsilon_{is} = \frac{P_s}{V_L \rho} = \frac{70147.54(cm^5)(s^{-3}) \cdot (g/ml)}{(1200ml) \cdot (1.005g/ml)}$$

$$\varepsilon_{is} = 58.17 (cm^5) \cdot (s^{-3}) / (ml)$$

$$\text{Therefore } \varepsilon_{is} = 58.17 (cm^5)(s^{-3}) / (cm^3) \text{ or } 58.17 cm^2/s^3$$

$$\underline{\varepsilon_{is} = 58.17 cm^2/s^3 = 0.0058 W/kg}$$

ε_t from equation (A2):

$$\varepsilon_t = 0.0024W/kg + 0.0058W/kg = 0.0082W/kg$$

Now the maximum shear stress can be determined

$$\tau_{\max} = 5.33 \cdot \rho \cdot (\varepsilon_t \cdot \nu)^{1/2} \quad (\text{A.1})$$

$$\begin{aligned}
\tau_{\max} &= 5.33 \cdot (1.005 \text{ g/ml}) \cdot ((0.0082 \text{ W/kg}) \cdot (0.0092 \text{ cm}^2/\text{s}))^{\frac{1}{2}} \\
&= (0.045 \text{ g/ml}) \cdot ((\text{W/kg}) \cdot (\text{cm}^2/\text{s}))^{\frac{1}{2}} \\
&= (0.045 \text{ g/cm}^3) \cdot ((\text{m}^2/\text{s}^3) \cdot (\text{cm}^2/\text{s}))^{\frac{1}{2}} \\
&= (0.045 \text{ g/cm}^3) \cdot ((\text{m}^{\frac{2}{2}}/\text{s}^{\frac{3}{2}}) \cdot (\text{cm}^{\frac{2}{2}}/\text{s}^{\frac{1}{2}})) \\
&= 0.045 \frac{\text{g} \cdot \text{m} \cdot \text{cm}}{\text{cm}^3 \cdot \text{s}^{\frac{3}{2}} \cdot \text{s}^{\frac{1}{2}}} \\
&= 0.045 \frac{\text{g} \cdot \text{m}}{\text{cm}^2 \cdot \text{s}^2} \\
&= 4.5 \text{ g} \cdot \text{cm}/\text{s}^2 \cdot \text{cm}^2
\end{aligned}$$

1 dyn = g.cm/s² and so the answer becomes

4.5 dyn/cm²

Appendix B:

Table B1: Chapter 4: Raw data for relative % of glycans present on rIgG1 harvested from 1 L Erlenmeyer shake and 1.7 L minifors reactor suspension cultures

	F(6)A1/A2	F6A2	M5	F(6)A2[6]G(4)1	F(6)A2[3]G(4)1	F(6)A2G2	A2G2	F6A2G2S1
<i>1 L erlenmyer shake flask culture</i>								
End of exponential phase	2.52 +/- 0.39	50.23 +/- 0.57	0.99 +/- 0.13	31.44 +/- 0.16	8.43 +/- 0.17	5.46 +/- 0.11	0.67 +/- 0.04	0.26 +/- 0.02
End of stationary phase	4.01 +/- 0.87	50.12 +/- 1.20	1.76 +/- 0.37	29.8 +/- 0.09	8.04 +/- 0.27	5.42 +/- 0.23	0.62 +/- 0.05	0.22 +/- 0.01
<i>1.7 L minifors reactor culture</i>								
End of exponential phase	2.93 +/- 0.51	57.64 +/- 2.72	1.28 +/- 0.17	25.72 +/- 1.71	7.67 +/- 0.57	3.77 +/- 0.53	0.72 +/- 0.1	0.25 +/- 0.02
End of stationary phase	4.18 +/- 0.39	56.74 +/- 2.77	2.15 +/- 0.70	24.55 +/- 1.54	7.44 +/- 0.66	3.71 +/- 0.45	0.96 +/- 0.1	0.27 +/- 0.01

Table B2: Chapter 5: Raw data for relative % of glycans present on rIgG1 harvested from 1.7 L minifors reactor suspension cultures and 1.7 L minifors reactor encapsulated cultures

	F(6)A1/A2	F6A2	M5	F(6)A2[6]G(4)1	F(6)A2[3]G(4)1	F(6)A2G2	A2G2	F6A2G2S1
<i>Suspension cells</i>								
End of exponential phase	2.93 +/- 0.51	57.64 +/- 2.72	1.28 +/- 0.17	25.72 +/- 1.71	7.67 +/- 0.57	3.77 +/- 0.53	0.72 +/- 0.1	0.25 +/- 0.02
End of stationary phase	4.18 +/- 0.39	56.74 +/- 2.77	2.15 +/- 0.70	24.55 +/- 1.54	7.44 +/- 0.66	3.71 +/- 0.45	0.96 +/- 0.1	0.27 +/- 0.01
<i>Encapsulated cells</i>								
End of exponential phase	2.77 +/- 0.49	59.27 +/- 3.95	0.94 +/- 0.04	25.5 +/- 2.71	7.46 +/- 0.81	3.32 +/- 0.74	0.55 +/- 0.74	0.24 +/- 0.04
End of stationary phase	3.16 +/- 0.08	54.09 +/- 5.45	2.71 +/- 0.17	27.28 +/- 4.90	7.38 +/- 1.03	4.18 +/- 1.41	0.93 +/- 1.41	0.3 +/- 0.03

Table B3: Chapter 6; Results Section 6.1: Raw data for relative % of glycans present on rIgG1 harvested from 1.7 L minifors reactor encapsulated batch and control-fed perfusion cultures

	F(6)A1/A2	F6A2	M5	F(6)A2[6]G(4)1	F(6)A2[3]G(4)1	F(6)A2G2	A2G2	F6A2G2S1
<i>Encapsulated cells perfusion minifors reactor</i>								
End of exponential phase	2.75	59.73	0.91	24.27	8.25	3.44	0.47	0.18
End of stationary phase	3.27	56.92	1.2	24.56	9.16	3.87	0.69	0.34
<i>Encapsulated cells batch minifors reactor</i>								
End of exponential phase	2.77 +/- 0.49	59.27 +/- 3.95	0.94 +/- 0.04	25.5 +/- 2.71	7.46 +/- 0.81	3.32 +/- 0.74	0.55 +/- 0.74	0.24 +/- 0.04
End of stationary phase	3.16 +/- 0.08	54.09 +/- 5.45	2.71 +/- 0.17	27.28 +/- 4.90	7.38 +/- 1.03	4.18 +/- 1.41	0.93 +/- 1.41	0.3 +/- 0.03

Table B4: Chapter 6; Results section 6.2: Raw data for relative % of glycans present on rIgG1 harvested from 1 L Erlenmeyer shake flask batch encapsulated cultures and 1.7 L minifors reactor batch encapsulated cultures

	F(6)A1/A2	F6A2	M5	F(6)A2[6]G(4)1	F(6)A2[3]G(4)1	F(6)A2G2	A2G2	F6A2G2S1
<i>Encapsulated cells batch shake flask</i>								
End of Exponential phase	2.96 +/- 0.22	54.19 +/- 0.55	1.09 +/- 0.1	27.83 +/- 0.16	8.77 +/- 0.30	4 +/- 0.13	0.78 +/- 0.03	0.39 +/- 0.02
End of Stationary phase	3.35 +/- 0.14	47.77 +/- 1.25	1.65 +/- 0.13	31.06 +/- 0.58	9.1 +/- 0.14	5.66 +/- 0.25	0.95 +/- 0.06	0.46 +/- 0.04
<i>Encapsulated cells batch minifors reactor</i>								
End of exponential phase	2.77 +/- 0.49	59.27 +/- 3.95	0.94 +/- 0.04	25.5 +/- 2.71	7.46 +/- 0.81	3.32 +/- 0.74	0.55 +/- 0.74	0.24 +/- 0.04
End of stationary phase	3.16 +/- 0.08	54.09 +/- 5.45	2.71 +/- 0.17	27.28 +/- 4.90	7.38 +/- 1.03	4.18 +/- 1.41	0.93 +/- 1.41	0.3 +/- 0.03

Table B5(i): Chapter 6; Results Section 6.3: Raw data for relative % of glycans present on rIgG1 harvested from 1 L Erlenmeyer shake flask control-fed perfusion culture in non-CO₂ incubator.

	F(6)A1/A2	F6A2	M5	F(6)A2[6]G(4)1	F(6)A2[3]G(4)1	F(6)A2G2	A2G2	F6A2G2S1
Perfusion encapsulated cells								
shake flask (non-CO₂)								
Beginning of perfusion	2.95 +/- 0.07	54.59 +/- 0.60	1.05 +/- 0.03	27.93 +/- 0.20	8.48 +/- 0.18	3.9 +/- 0.11	0.74 +/- 0.03	0.37 +/- 0.04
End of increasing cell number	4.94 +/- 0.55	43.25 +/- 0.73	2.83 +/- 0.24	31.86 +/- 0.90	8.91 +/- 0.33	6.73 +/- 0.27	1.01 +/- 0.05	0.46 +/- 0.04
End of stationary phase	5.25 +/- 0.05	41.49 +/- 0.61	3.27 +/- 0.08	32.3 +/- 0.45	8.76 +/- 0.16	7.45 +/- 0.20	1.03 +/- 0.03	0.45 +/- 0.03

Table B5(ii): Chapter 6; Results Section 6.3: Raw data for relative % of glycans present on rIgG1 harvested from 1 L Erlenmeyer shake flask control-fed perfusion culture in 5 % CO₂ incubator.

	F(6)A1/A2	F6A2	M5	F(6)A2[6]G(4)1	F(6)A2[3]G(4)1	F(6)A2G2	A2G2	F6A2G2S1
Perfusion encapsulated cells								
shake flask (CO₂)								
Beginning of perfusion	3.32 +/- 0.17	52.58 +/- 0.48	1.27 +/- 0.07	28.61 +/- 0.29	8.92 +/- 0.14	4.20 +/- 0.15	0.74 +/- 0.04	0.36 +/- 0.03
End of increasing cell number	5.42 +/- 0.22	41.42 +/- 0.69	3.3 +/- 0.11	32.43 +/- 0.57	9.21 +/- 0.10	6.66 +/- 0.24	1.08 +/- 0.05	0.47 +/- 0.03
End of stationary phase	11.35 +/- 0.47	61.33 +/- 1.09	3.50 +/- 0.16	16.42 +/- 0.62	4.75 +/- 0.22	2.05 +/- 0.13	0.46 +/- 0.04	0.15 +/- 0.02

