Process Physiology of Antibody Producing Mammalian Cell Lines in Batch and Extended Batch Cultures

Nurzila binti Ab. Latif

Strathclyde Institute of Pharmacy & Biomedical Sciences

A thesis submitted to the University of Strathclyde in part fulfilment of the regulations for the degree of Doctor of Philosophy.

2018

Copyright Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

The copyright of this thesis belongs to the author under the terms of the United Kingdom Copyright Acts as qualified by University of Strathclyde Regulation 3.50. Due acknowledgement must always be made of the use of any material contained in, or derived from, this thesis.

Signed: 

Date:

This dissertation is dedicated to my late father, Ab. Latif Ibrahim who always motivates and inspired me during my study. I love you.

Acknowledgements

It is a pleasure to thank the many people who made this thesis possible.

First and foremost, I would like to thank my supervisors: Professor Brian McNeil and Professor Linda Harvey for giving me the opportunity to work with them. They have given me the guidance, advice and penetrating criticism throughout my study, which has helped in building my practical and intellectual skill.

My special thank goes to all my friends and colleagues within SIPBS, but in particular those in the Strathclyde Fermentation Centre: Dr Mariana Fazenda, Dr Peter Gardner, Dr Chiara Viegelmann, Dr Laura Jeffrey, Dr Wan Al Qadyr Imad Bin Wan Mohtar, Dr Scott Parker, Joana Faustino, Ellis Robb and Walter McEwan, who always provide hands and knowledge throughout my PhD.

Thank you very much to the sponsor of my PhD, Ministry of Higher Education Malaysia and University Technology and to Lonza Ltd and Thermo Fisher Scientific for providing me with all the materials to carry out this research.

I would also like to express my special appreciation for my beloved parents and my family as well as Malaysian Glasgow Community for their great support and encouragement from the day I arrived in Glasgow.

And finally, I would like to express my eternal gratitude to my beloved husband, Abdull Rahman, for all the tolerance and understanding he has shown me, as well as his incredible ability to encourage me through the finishing phases of writing.

Thank you my children, Aliesya Zara and Afie Firdaus, for their love and for being by my side through ups and downs. This thesis is a gift to you.

Content

List of Figures

List of Tables

Abbreviations

A7r5	rat aortic smooth muscle cells
ACE	artificial chromosome expression
AMBIC	ammonium bicarbonate
BHK	baby hamster kidney
cAD-MSCs	Canine adipose-derived mesenchymal stem cells
CD	Chemically Defined
CDR	complimentary determining region
CHO	Chinese Hamster Ovary
CO_2	Carbon Dioxide
DHFR	dihydrofolate reductase
DMSO	Dimethyl Sulfoxide Dissolved Oxygen
dO_2	Dissolved Oxygen
EFA	Efficient Feed A
EFB	Efficient Feed B
ELISA	Enzyme-Linked Immunosorbent
GCMS	gas chromatography mass spectrometry
GS	Glutamine synthetase

H_2O_2	Hydrogen Peroxide
HAHA	human anti-humanized antibodies
HAMA	human anti-mouse antibodies
HEK-293	human embryonic kidney
HMDB	Human Metabolome Database
PER-C6	human-retina-derived cells
HPLC	High-Performance Liquid Chromatography
IgG	Immunoglobulin G
IPA	isopropyl alcohol
IPTG	isopyl-beta-D-thiogalactopyranoside
KOH	potassium hydroxide
KEGG	Kyoto Encyclopedia of Genes and Genome
LCMS	liquid chromatography mass spectrometry
LDH	Lactate dehydrogenase
LOD	limit of detection
mAb	Monoclonal Antibody
MDCK	Madin-Darby canine kidney
MFA	metabolic flux analysis

MeOH	methanol
MWP	24-microwell plate
miRNA	Micro Ribonucleic Acid
NaCl	Sodium Cholride
NaOH	sodium carbonate
NS0	mouse myeloma
NIR	near infrared
NMR	nuclear magnetic resonance
MSX	Methionine Sulfoximine
NIR	Near Infrared
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
PAT	Process Analytical Technology
PC	Principal Component
PCA	Principal Component Analysis
PyLT	polyoma virus large T-antigen
PDH	pyruvate dehydrogenase
RNA	Ribonucleic Acid
SD	Standard deviation

TCA	tricarboxylic acid
tRNA	transfer ribonucleic acids
v/v	Volume/Volume Percent
w/v	Weight/volume Percent
YE	yeast extract
α-KG	alpha-ketoglutarate
μ	Specific growth rate
^{1}H-NMR	^{1}H-nuclear magnetic resonance
ε	energy dissipation rate

Abstract

The increasing usage of monoclonal antibodies (mAbs), often expressed in Chinese Hamster Ovary (CHO) cells, for human therapy, has led to a focus on rational approaches to speed the development of cost-effective and highly productive cell lines. Understanding the process physiology of industrial CHO cell lines is important to making significant progress in cell line and culture development. In this study, which was underpinned by the supply of several industrial CHO cell lines by an industrial collaborator (Lonza Biologics) and newly developed industrial CHO media by another industry partner (Thermo Fisher Life Sciences), the effects of several process variables including different clones of cells, passage number, scale of culture, culture medium, feed supplements and culture modes (batch and fed-batch) on cell line physiology were investigated. GS-CHO 42 cell lines a high monoclonal antibody producer with low passage number (4) were observed to give better results compared to a less productive cell lines and higher passage numbers. In addition, three commercially available, chemically defined CHO cell culture media (CD-CHO, CD-OPTICHO and Dynamis) and two different types of feed supplements, CHO CD Efficient Feed A (EFA) and CHO CD Efficient Feed B (EFB) were evaluated in batch culture and fed-batch culture using the GS-CHO 42 cell line passage number 4. Cell culture in shake flasks and bioreactors showed clear effects of culture system on process physiology. In contrast, concentrated feed supplements did not help to increase the cell concentration and antibody titre. Amongst the three media tested, CD-CHO medium was found to be the best culture medium for GS-CHO 42 passage number 4 based upon the cell density, viability and Immunoglobulin (IgG) titre produced.

A metabolomics study was carried out on samples from these cultures to observe the metabolic profiles under different culture conditions. Statistical analysis with Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were performed using SIMCA version 14.0 to view the underlying global structure of the expression data. The results show that different metabolites were present under different culture conditions (different culture medium, scales and mode of culture) and were associated with different physiological behaviour of GS-CHO cell cultures. By using the results of this study, several bioprocessing strategies including medium improvement, feeding strategy and downstream processing can be potentially implemented to achieve efficient CHO culture system. Nevertheless, more detailed studies are warranted to confirm and complement the existing information. The results in this work show how important process related information can be obtained with univariate and multivariate process analysis methods. Especially in cell culture process development, which is characterized by lengthy run times, a large number of influential and mutually interacting factors, as well as high-cost raw materials and process analytics, multivariate data analysis represents an attractive and versatile tools in process development.

Chapter 1

Introduction

1.1 Thesis objectives and structure

This thesis aims to increase our understanding of the process physiology of industrial CHO cell lines (supplied by Lonza Ltd) in batch and extended batch cultures. One key aspect of this involves metabolic characterisation of these cultures using metabolomic analysis by mass spectrometry. This will permit better insights into process effects upon metabolic profiles and the relationship of these with product titre and quality.

Objective 1: Characterise the physiology of industrial CHO cell lines at different growth phases in a shake flask batch culture system.

In chapter 3, two types of cell lines which are a high producer of IgG and low IgG producer will be cultured in two different commercially available culture media. Samples will be taken every 24 hours for physiological studies. Study of different passage number for each cell lines will also be conducted. Samples will be processed for growth profile and extracellular metabolite analysis using a Biochemistry Analyser (YSI Incorporated, Hampshire, UK).

Objective 2: Characterise the physiology of industrial CHO cell lines cultured in bioreactors using batch culture mode.

In chapter 4, the effect of three different commercially available culture medium on cell physiology and product quality of CHO cells culture will be observed. Comparison

between small scale (shake flask) and a larger scale culture (bioreactor) will also be done. It also aims to develop a relevant feeding strategy for extended batch cultures.

Objective 3: Characterise the physiology of industrial CHO cell lines cultured in bioreactor using fed batch culture mode.

In chapter 5, the effect of different media on cell physiology and product quality of CHO cells culture will be observed in fed batch mode. Comparison between batch and fed batch culture will also be done.

Objective 4: To monitor the intracellular metabolites that may be present during the culture process of industrial CHO cell lines cultured in various conditions and environment using metabolomics technique.

In Chapter 6, mass spectrometry will be used to look at the different metabolites that are present during the culture of CHO cells in different culture conditions and environments.

Objective 5: To draw clear conclusions from the overall project and make recommendations for future work.

1.2 Biopharmaceuticals

According to Walsh (2014), the number of approved biopharmaceuticals marketed in the United States and/or EU now stands at 212 and these includes monoclonal antibodies (mAb) and biosimilars. Over the past four years, biopharmaceuticals from therapeutic antibodies has seen an increase in its growth and sales (Aggarwal, 2014). The term "biopharmaceuticals" which are related with protein or nucleic acid-based pharmaceutical substance used for therapeutic or *in vivo* diagnostic purposes, which is produced from biological sources is often used interchangeably with "biologicals", "biologics" and "biological products' (Castilho, 2016). Biopharmaceuticals are also known as protein based products that are used in helping human therapeutic and diagnosis in the medical field. The illnesses and conditions that have been helped by the biopharmaceutical products are wide ranging and have different physiological role in human body including as catalysts, receptors, membrane channels, macromolecule carriers and cellular defence agents (Jozala *et al*., 2016). Recombinant DNA and hybridoma technologies are used to engineer biological systems to produce biopharmaceutical products including recombinant forms of natural proteins (human growth hormones, cytokines and insulin), derivatives of natural proteins (cancer cell proteins and IgG fusion proteins), viral and plasmid vectors and *in vivo* diagnostic and therapeutic monoclonal antibodies (Zhu, 2012).

The categories of biopharmaceutical products include monoclonal antibodies, recombinant proteins, nucleic acid, hormones and growth factors (Berlec & Strukelj, 2013). Insulin was the first product that gained approval from the FDA for the use in human treatment, which is for used for treatment of diabetes (Ferrer-Miralles *et al*.,

2009). Nowadays, many kinds of new products that can aid in the medical field have been recognized and registered. Many researchers have been analysing new biopharmaceutical products. The five major fields of medicine in which biopharmaceuticals are used are cancer, diabetes, growth disturbances, haemophilia and hepatitis (Walsh, 2010).

1.3 Monoclonal Antibodies

Monoclonal antibodies were first introduced in 1975 by Kohler and Milstein where they describe the production of murine monoclonal antibodies (mAbs) from hybridomas. According to Kunert and Reinhart (2016) monoclonal antibodies are the largest group of recombinant proteins used not only for human therapy but also for in vivo imaging of different types of diseases. Walsh (2014) reported that over the past four years, mAbs recorded the highest number of approval with 17 from 54 biologics approved in the United States and European Union (EU; Brussel). In addition, in the year 2012, mAbs were recorded as the highest selling biopharmaceuticals with total revenue of $24.6 billion in the United States's biotechnology sector (Aggarwal, 2014). Nevertheless, the murine mAbs have two major problems for use in humans, first, human receptors will not bind well to the receptors of a murine immunoglobulin and second, humans will also develop human anti-mouse antibodies (HAMA) which lead to an inherent immunogenicity in man. Therefore the development of chimeric (mouse-human) mAbs took place, these are approximately 65% human origin and thus less immunogenic then murine mABs. However, these chimeric may also prompt human anti-chimeric antibodies and this then led to the production of humanized

mABs. Humanized mABs have more than 90% of human origin but patients can still have human anti-humanized antibodies (HAHAs). As the results, fully human mABs are being developed and currently there are 17 unconjugated human antibodies approved from FDA for clinical use. Figure 1.1 shows the schematic classifications for the antibodies according to their sequence source.

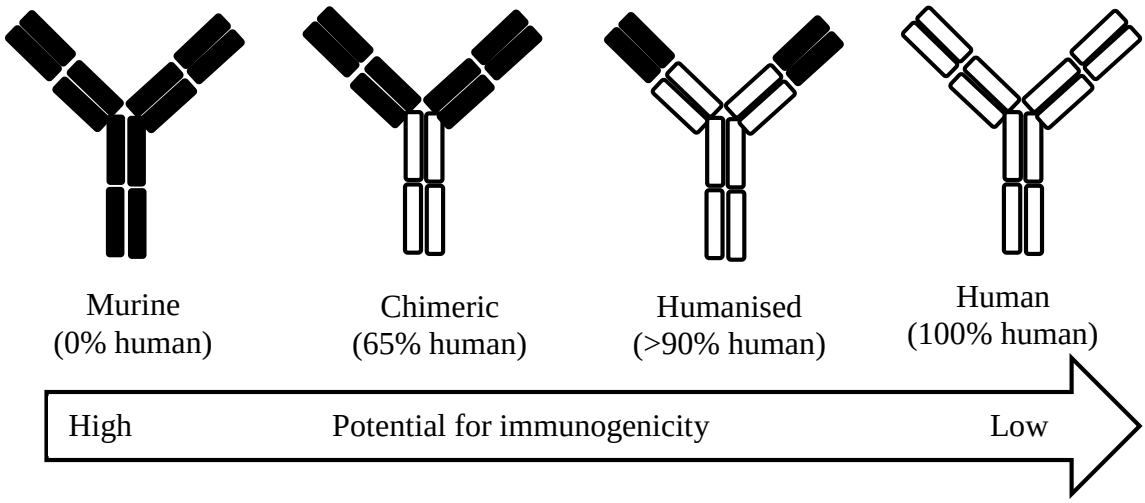


Figure 1.1 Schematic classification of antibodies according to their complementary sequence source. A: murine, B: chimeric, C: humanised, D: human.

1.4 Expression systems

In order to produce biopharmaceuticals, a host system is required. Examples of host systems, also known as expression systems are microorganisms, animal cells and also plant cells. Choices of expression systems are based on the product aim. Among the most widely used expression systems are *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia pastoris* and Chinese Hamster Ovary (CHO) cells (Berlec & Strukelj, 2013).

Escherichia coli is the most widely used bacterial expression system. It is a well-studied organism and well developed in term of its physiological properties. *E. coli* has several beneficial properties including rapid cell growth, convenience for genetic manipulation, ease in scale up, fast generation of a recombinant protein and low cost for large production (Lee *et al*., 2013, Busso *et al*., 2011, Song *et al*., 2012, Vernet *et al*., 2011, Khow and Suntrarachun, 2011). Despite all the advantages of *E. coli*, there are also several drawbacks for using it as an expression system. One of the problems that affects *E. coli* as an expression system is protein folding. Figure 1.2 shows that recombinant proteins are expressed in three ways from *E. coli*, which are, into the cytoplasm, into the periplasm and into the growth medium (Berlec and Strukelj, 2013). Expression into the cytoplasm is the most frequently used because it is the most straightforward method, and the production yields are usually high. The accumulation of heterologous protein, especially eukaryotic and disulphide bond-rich proteins often results in the formation of inactive inclusion bodies either in the cytoplasm or periplasm that lead to solubility problems for the recombinant protein expressed (Lee *et al*., 2013 and Song *et al*., 2012).

According to Vernet *et al*., (2011), although some research has been successful on protein purification from inclusion bodies, yields are typically low and a robust re-folding protocol must be developed for each protein. Other than that, the absence of particular post-translational modification, for example glycosylation, within *E. coli* is also a major problem (Busso *et al*., 2011). Glycosylation is an enzymatic process where glycans are added to specific amino acids in the polypeptide chain. It affects protein folding, stability, solubility, protein-protein interactions and *in vivo* bioavailability, biodistribution, pharmacokinetics and immunogenicity (Croset *et al*., 2012). Khow and Suntrarachun (2011) noted that *E coli* (a prokaryotic organism) lacks the cellular organelles that are essential for the glycosylation process.

In addition codon usage is another problem with the *E. coli* expression system. The codons appearing in *E. coli* genes are different from those occurring in human genes, and this is directly related to the abundance of specific transfer ribonucleic acids (tRNAs). As a result, heterologous genes that contain codons rarely used in *E. coli* may be inefficiently expressed in *E. coli* and may cause translation errors thus reducing the yield of expected protein versions (Khow and Suntrarachun, 2011 and Ferrer-Miralles, 2009).

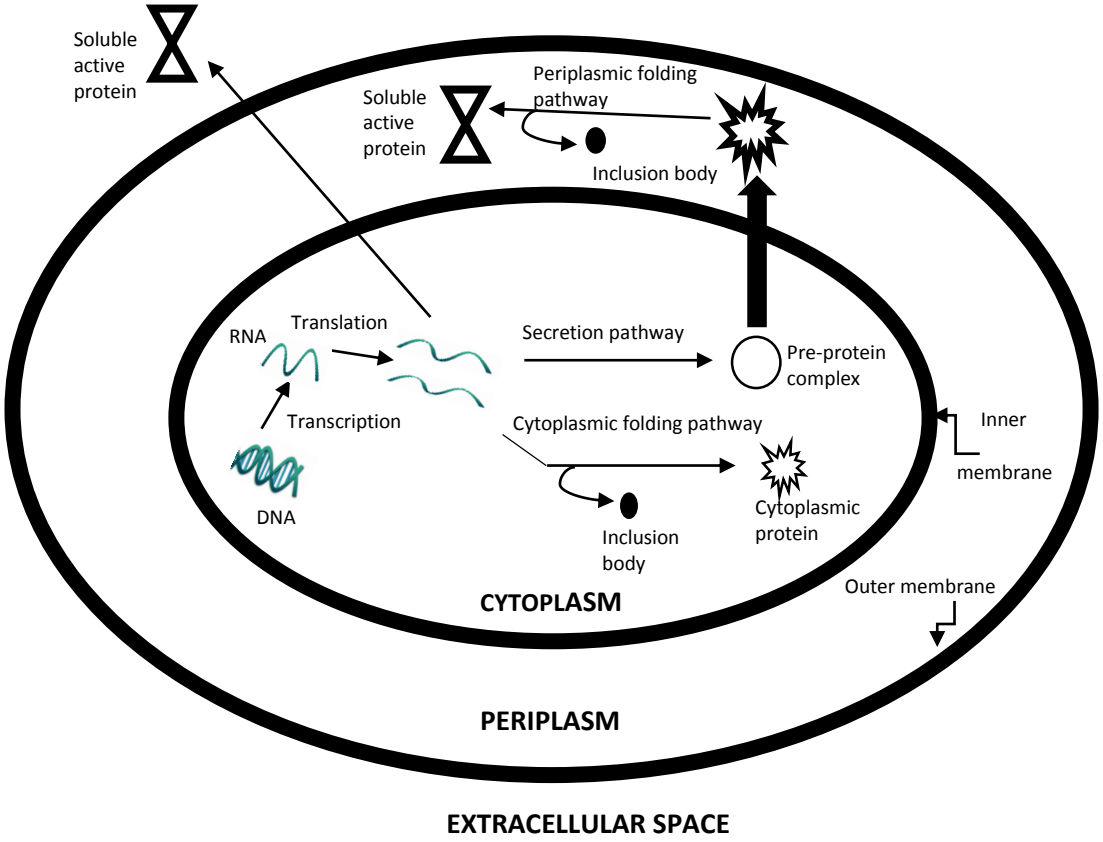


Figure 1.2 Protein expression recovery sites in *E. coli*; cytoplasmic, periplasmic and growth medium.

There may also be issues with the promoters used for protein expression. Ideally a strong promoter that controls the transcription of the target gene is needed. It should be capable of initiating a high level of transcription, tightly regulated, transferable to a number of *E. coli* strains and inducible in a simple and cost-effective manner (Berlec and Strukelj, 2013). Promoters may be induced by thermal change or with the addition of chemicals; the most common inducer is the molecule isopropyl-beta-D-thiogalactopyranoside (IPTG). Nevertheless, IPTG has its own drawbacks because it

is not suitable for large scale production of human therapeutic protein due to its toxicity and also its high price (Berlec and Strukelj, 2013 and Khow and Suntrarachun, 2011).

Yeast species have also been popular industrial hosts for recombinant protein production because they have the advantages of unicellular organisms, which are ease of genetic manipulation and rapid growth. They also have the ability to perform some eukaryotic post-translational modifications. Yeast expression system have been used widely in the biopharmaceutical industry because unlike other complex eukaryotic organisms, yeast can be grown in cheap and defined media, thus making the process economical, can rapidly reach high cell densities, produce high protein titres and do not contain pyrogens, pathogens or viral inclusions bodies which is a site of viral multiplication in a bacterium or other eukaryotic cell *(*Çelik and Çalık, 2012; Martínez *et al*., 2012). In addition, the yeast cells are less sensitive than some animal cell lines since the cell wall makes them more resistant to shear stress during the production process (Martínez *et al*., 2012). Among the two most popular yeasts that have been used are *Saccharomyces cerevisiae* and *Pichia pastoris*. Both have several advantages according to the product of interest. According to Huang *et al*., (2010), recombinant therapeutics approved by the Food and Drug Agency (FDA) and the European Medicines Agency (EMEA) from microbial eukaryotic cells are almost exclusively produced by *S. cerevisiae*. Despite all the advantages, culturing using yeast does have its limitations. Even though they are eukaryotic systems, their glycosylation of proteins may be different to that performed in mammalian culture (Martinez *et al*., 2012).

Another category includes both individual animal and individual plant expression systems. A variety of transgenic animal species are being used to produce recombinant proteins. The idea of it is to overcome the limitations facing traditional

recombinant pharmaceutical protein production systems. One example is to target the expression of the desired protein to the mammary gland using regulatory elements derived from a milk protein gene and then collect and purify the product from milk (Freitas *et al*., 2012). Rabbit also has been used as transgenic production platform that has the advantage of a relatively short gestation period and the possibility to be maintained in a closed environment to minimize the introduction of adventitious agents (van Veen *et al*., 2012). However, none of the products have reached the market yet due to several technical problems. Using transgenic animal expression systems is very challenging because they are high maintenance with longer production periods yet producing low yields (Forabosco *et al*., 2013). On the other hand, transgenic plant expression system are more flexible and useful. The heterologous proteins expressed can be localized to different organs of the plant. Other than that, some proteins of interest can also be expressed at specific growth stages. One of the most important aims in the biopharmaceutical industry is to reduce cost especially in the production phase, transgenic plants can easily be grown in fields and so reduce the cost relative to such proteins produced in a bioreactor. Lastly, the protein products that come from plants are extremely stable, readily stored, and easily extracted and purified (Hefferon, 2012). Anyway, despite all the advantages, there are also a few limitations, which are low transformation and expression efficiencies in plants, difficulties in the establishment of plant regeneration systems, a different glycosylation pattern from animal derived cell expression systems and also some controversial safety aspects (Hefferon, 2012).

Nowadays, mammalian cells are being widely used for recombinant protein production. There are many advantages of mammalian cells compared to the systems

discussed above. Mammalian cells can easily be grown in suspension conditions which will ease the process of scaling up (Carvalho *et al*., 2017). Other than that, mammalian cells may be capable of correctly doing the post translational modifications which are required for the final functional recombinant protein. Modification, such as proteolytic processing and disulphide bond formation, which is the part of the primary structure of a protein, can to some extent be carried out by microorganisms and yeast systems, but at present the glycosylation process can only be done effectively in the mammalian cells system (Zhu, 2012). This process, which involves addition of a glycosyl group to a protein in order to form a glycoprotein, is important in protein folding, protein targeting and trafficking, ligand recognition and binding, biological activity, stability, protein half-life and evoking an immune response (Croset *et al*., 2012). Examples of mammalian cells that have been used in the production of biopharmaceuticals are Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, human embryonic kidney (HEK-293) cells, human-retina-derived cells (PER-C6) and mouse myeloma (NS0) cells (Zhu, 2012). Among all of these, CHO cells have been producing the majority of approved proteins (Badsha *et al*., 2016; Sellick *et al*., 2015; Kildegaard *et al*., 2013).

1.5 Chinese Hamster Ovary (CHO) cells

Chinese hamster ovary cells were first isolated by Dr. Theodore T. Puck of the Department of Medicine at the University of Colorado in 1957 (Robinson, 1958). His team had successfully grown Chinese hamster and described the cell line as tough and reliable. Cultures from the ovary had been maintained in continuous cultivation for more than 10 months with no decrease in growth rate or change in cellular or colonial morphology (Robinson, 1958). CHO cells have contributed to many basic biomedical research studies. Despite many failures due to their diploid state, which consists of two copies of each chromosomes; CHO became an established cell line because many of their genes are hemizygous. The genotype for a single gene in a diploid organism is often referred to as being either homozygous, heterozygous or hemizygous. Homozygous means that both alleles are the same for example (BB or bb). Heterozygous means that the two alleles are not the same; (Bb or B1B2). While the term hemizygous means that there is only one allele (instead of two) in a diploid cell. The expression of a pharmaceuticals product in diploid cells requires both copies of the gene be converted to the mutant form, which is difficult in a CHO cell culture. Research by Campbell and Worton (1979) showed that CHO may have lost or inactivated one copy of many of its genes and this results in functional hemizygosity. In addition, Simon *et al*., (1982) also proposed that gene inactivation activity in the CHO cells could be used to solve the problem of diploid CHO cells. As a result, CHO cells were used widely in biomedical and biopharmaceutical studies. One of the important findings with CHO cells, is the recognition of a mutant strain for example an auxotroph that offers the possibility of using the cells for industrial production. The findings facilitate the industrial production of biopharmaceuticals because cultured

CHO cells are able to live longer and only require a particular nutrient to maintain their growth. Examples of mutants that have been isolated and used are dihydrofolate reductase (DHFR) and Glutamine synthetase (GS) (McAtee *et al*., 2014) The DHFR selection system allows selection of firmly transfected cell lines and efficient gene amplification which results in higher titer of cell culture product (Florin *et al*., 2011). While the advantage of using a GS expression system is that this system catalyses the formation of glutamine from glutamate and ammonia thus results in a low level of toxic ammonia accumulation in the culture (McAtee *et al*., 2014).

Other than that, the great adaptability and easy handling of CHO cells in culture result in them being widely used in the biopharmaceutical industry (Castilho, 2016). CHO cells also are able to grow in suspension condition, which is a very important aspect for the scaling up in the industrial sector. Last but not least, CHO cells are amenable to genetic modifications allowing easy introduction of foreign DNA and expression of large amounts of desired protein (Jenkins *et al*., 2008). Recombinant proteins produced from CHO cells have been proven to be compatible and bioactive in humans. CHO cells also been confirmed as a safe expression system in the production of biopharmaceuticals. During the downstream process, contaminant levels from CHO cells culture were low, and according to Jayapal & Wlaschin (2007), a study in 1989 confirmed that human pathogenic viruses including HIV, influenza, polio, herpes and measles do not replicate in CHO.

Despite all the advantages of CHO cells, there are also some limitations or drawbacks. First, is choosing the best clone in order to produce high yield of the desired protein. Research on using a gene targeting procedures have been done on CHO cell lines. One of the procedures is by using engineered chromosomes and the

example of the system used is the artificial chromosome expression (ACE) system that targeted transfection of cells containing mammalian-based artificial chromosomes with multiple recombination acceptor sites. This system has been demonstrated in several case studies covering the generation of CHO cell lines expressing monoclonal antibodies (Kennard, 2011).

Secondly, optimization of culture medium is very crucial in order to achieve high cell viability and productivity. Fetal bovine serum has traditionally been used in the cell culture medium because it has many growth factors that promote cell growth and also protein production. Despite the advantages, serum also has many drawbacks, such as unknown components that may lead to contamination and give difficulties in the downstream process. Therefore, many studies have been done in order to replace serum in a culture medium (Butler, 2005 and Costa *et al*., 2012). New clones of CHO cells also need to have their own research team in order to find the optimal medium for it.

Another major problem in CHO cell cultures is programmed cell death which includes apoptosis and autophagy. Research findings showed that controlling of the programmed cell death will lead to a higher cell viability and protein production. Lee and Lee (2012) established a rapid and easy monitoring method for the simultaneous detection of apoptosis and autophagy in CHO cells. They studied the apoptosis and autophagy induction, their relative levels, and temporal changes in CHO cells under starvation conditions in order to develop a scheme for anti-cell death engineering and the genetic regulation of programmed cell death pathways. Their data are very useful for the potential application to long term CHO cell cultures and also can be applied to other mammalian cell lines.

Other than that, stirring and sparging are essential in a cell culture condition in order to supply oxygen and nutrient to the cells. However, both are sources of hydrodynamic stress that could cause cell damage or affect cell's performance. Cell damage in the culture was caused by the bursting of gas bubbles produced from the sparging process. The hydrodynamic stress from both stirring and sparging also triggered the necrosis and apoptosis of cells. Several studies have been done to monitor the stirring and sparging effects on CHO cell culture conditions (Sieck *et al*., 2012, Nienow *et al*., 2013)

Carbon sources in a cell culture medium are derived from glucose, glutamine or glutamate. However, glucose will produce lactate while glutamine and glutamate will produce ammonium and both by-products can cause harmful effects on the cells and lead to a low protein productivity. In 2011, Berrios *et al.* studied the replacement of glucose using mannose. They observed the increased biomass, lower specific hexose consumption rates, and also lower specific production rates of lactate, an undesirable by-product in a culture. Moreover, the volumetric productivity and end product level were also increased.

Chabanon *et al*. (2008) studied the influence of different enzymatic hydrolysis processes on the characteristics of rapeseed hydrolysates and secondly the effect of CHO cell growth in serum-free media supplemented with those hydrolysates. Their results showed that hydrolysates from different degree of hydrolysis gave different results. Hydrolysates from extensive hydrolysis, corresponding to a major low molecular size peptides content, usually allowed an increase of the maximal cell density. They concluded that total substitution of proteins (transferrin, albumin and insulin) in the cell culture medium by some rapeseed hydrolysates appeared to be a

promising alternative to improve the cell growth in protein-free media. Other than that, hydrolysates from yeasts or plant proteins gave promising capacity to improve cell growth and recombinant protein productivity. However, they are very complex and contain many unknown peptides that will cause difficulty in the downstream processing.

Mosser *et al*. (2012) studied on the effect of yeast extract (YE) composition on CHO cell growth and productivity. They used chromatographic strategies to study the physicochemical properties of molecules in yeast extract mixtures that can improve CHO cell cultures. Their findings supply the knowledge of YE contribution to animal cell culture and also to evaluate fractionation strategies to simplify the complex mixture. They also found that cationic peptides from YE might improve CHO cell growth by facilitating nutrient transfer into the cells. Xing *et al*. (2011) used metabolic flux analysis (MFA) to identify amino acids that are limiting or in excess in a batch culture. The data were used to modify the feed media by increasing concentrations of the limiting amino acids and reducing concentrations of excess amino acids.

Wang *et al*., (2010) re-engineered the CHO cells producing a therapeutic monoclonal antibody directed against the glycoprotein receptor of human platelets with the 30Kc6 gene and 30Kc19 gene to investigate their anti- apoptotic effect. Their observation showed that 30Kc6 was much more effective at increasing cell viability and mAb production than 30Kc19, and also could prevent apoptosis by maintaining the mitochondrial membrane potential. Based on these results, 30Kc6 holds great promise for use as a potent anti-apoptotic gene in cellular engineering strategies to increase monoclonal antibody production. Kim *et al*., (2012) also did research on controlling apoptosis of CHO cell in a culture system. They used two effective cell

engineering strategies which are combining of polyoma virus large T-antigen (PyLT) and Bcl-xL in CHO DG 44 cells. The CHO-DG44 cells were first engineered to over-express Bcl-xL for anti-apoptosis, and then engineered to express PyLT for the episomal replication with the use of a DNA expression vector encoding PyOri, EBNA-1, and OriP. Rajkumar *et al.*, 2005 showed that the episomal or extrachromosomal vectors persist in the nucleus without the requirement to integrate into the host genome, hence avoiding the recent concerns surrounding the genotoxic effects of vector integration. This replication system is reported to allow more plasmids copies to persist in the transfected cells throughout the production phase leading to a significant increase in transgene expression (Kunaparaju *et al.*, 2005).

Studies on cell culture conditions also will help in increasing the cell growth and productivity. Becerra *et al.*, (2012) reviewed studies of the effect of mild temperature on CHO cell growth and its protein production. They found that decreasing the temperature of a culture from normal temperature, 37^{o}C to 28^{o}C, a mild hypothermia condition, allows an increase in the specific productivity of protein production in CHO cell culture, and no significant changes in the glycosylation profile were found.

Lactate is a by-product from the use of glucose as a carbon source in a cell culture. Intracellular concentration of pyruvate in a mammalian cell culture is an important influence on the production of lactate. Lactate dehydrogenase (LDH) catalyzes the pyruvate into lactate, while pyruvate dehydrogenase (PDH) catalyzes the reaction converting pyruvate to acetyl-CoA which will enter the tricarboxylic acid (TCA) cycle. Both enzymes determine whether the consumed carbon source is excreted as lactate after oxidation through glycolysis or metabolized further in the

TCA cycle. Zhou *et al*., (2011) knocked down the gene expression of lactate dehydrogenase A (LDHa) and pyruvate dehydrogenase kinase (PDHKs) to investigate the effect on lactate metabolism and protein production using a single targeting vector carrying small inhibitory RNAs (siRNA) for LDHa and PDHKs. Their research found that lactate production in CHO cell culture was reduced and antibody production was increased by this strategy.

Usually, stirring and sparging are often found in large scale of culture systems. It may be hard to predict how the stirring and sparging regime in small bioreactors will be translated into larger scale production conditions and this may lead to challenges in process scale-up. Therefore, Sieck *et al.*, (2012) established a Scale-Down Model of production bioreactors to test the performance and robustness of a new CHO cell line at various levels of hydrodynamic stress comparable to those found in a production scale bioreactor. They found that the cells are robust against hydrodynamic stress with respect to cell growth and fundamental metabolic rates. However, a slight decrease of monoclonal antibody production was observed with increasing hydrodynamic stress and they suggested that DNA damage is induced by the applied hydrodynamic conditions. Nienow *et al.*, (2013) studied the impact of spatial variations in pH and nutrients on CHO cells as found in a typical industrial scale bioreactor, and the impact of fluid dynamic stresses associated with agitation. The first study failed because it required cells to be circulated throughout the culture using a peristaltic pump and this process shorten the culture time thus result in decreasing of productivity of cells compared to control culture without recirculation. While in the second study they found no significant effect on cell growth and productivity by the dynamic stress introduced. However from their findings, it is crucial to have a reliable scale down test

for the assessment of cell robustness as new cell lines are introduced due to the specific behaviour of each cell lines.

Micro well plates are useful in the study of a scale down process for culture cell because they save lots of cost and are easy to use. However, one limitation of using micro well plates, is the poor mixing that will negatively affect cell growth, product quality and quantity. This is because mammalian cells tend to aggregate and accumulate at the bottom of the well, therefore, this will cause an error during sampling and cell counting which an important part in cell culture is. Wen *et al.*, (2012) described a novel 24-microwell plate (MWP) for suspension mammalian cell culture. The performance of 24-MWP with different patterns of static mixers was evaluated. They compared the data from the 24-MWP with cells culture in a normal commercial micro well plates and also culture in spinner flask. The results verified the scalability of the 24-MWP, and its potential use as a miniature cell culture device in high through-put cell culture applications.

1.6 Metabolomics

Metabolites are compounds that are found inside cells and their external environment that are involved in the biochemical reactions in cells. It is difficult to know the exact metabolites that are produced during a particular culture phase, and also to understand if those metabolites affect cell growth but most importantly the antibody production in cultured mammalian cell systems. Nowadays, great attention has been put into the emerging field of 'omics' with the aim of to achieving improvements in cell culture systems, and moreover antibody production. Metabolomics is one of the major 'omics' techniques that have gained attention from many researchers. The measurement of metabolites within an organism and the process will complete the story of the 'omics' research strategy for a particular cell lines (Figure 1.3). Genomics is where the whole genome (DNA) and its function being investigates. DNA will then being transcripted into mRNA which involves the Transcriptomics process. After that, Proteomics will analyse the protein expression profile after protein being translated from mRNA. Last but not least, Metabolomics will examine the metabolites that being expressed.

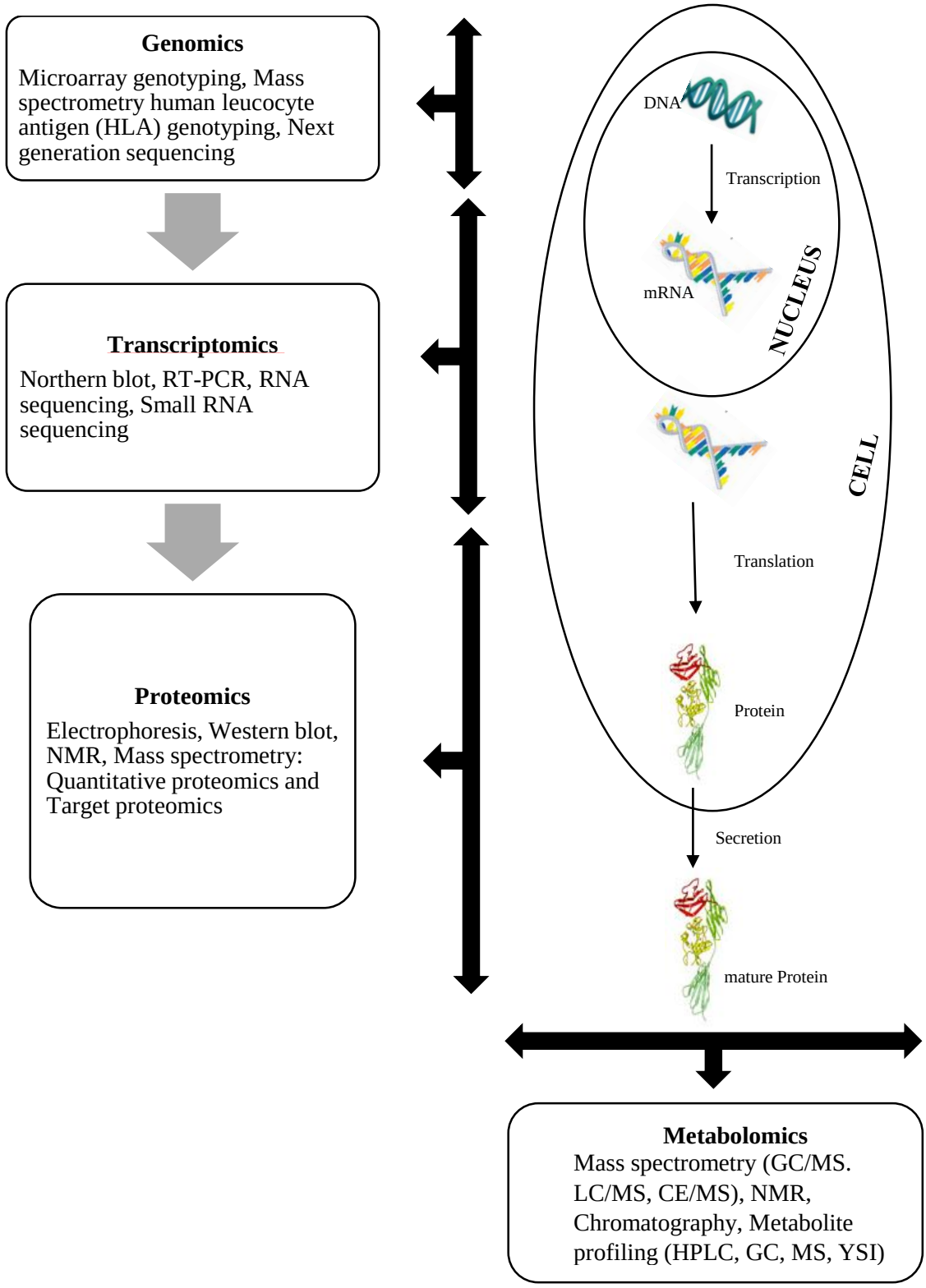


Figure 1.3 Research strategies in 'omics' with their selected research instruments. Adapted from (Datta *et al*., 2013; Tuñón *et al*., 2010).

Research has shown that it is important to monitor the concentration of intracellular metabolites during the culture process (Floris *et al.*, 2017; Karst *et al*., 2017; Sellick *et al*., 2015). Requirements of nutrients and production of metabolites are different throughout the culturing process. Research by Sellick *et al*., (2011) shows that changes in culture conditions and environment have a major effect on product quality. They found some nutrients that were limiting for the cultured cells, and this affected antibody titre. From the metabolite analysis of the culture supernatant, the study found that reductions in several nutrients and amino acids are the reason for the transition from one phase to another in the culture system. They observed that exhaustion of asparagine, aspartate, glutamate, and pyruvate was the reason of the culture going into stationary phase from the exponential phase. Subsequently, the move into decline phase from stationary phase was because of the exhaustion of glucose, leucine, lysine, and serine. From these results they tested feeding the culture with those nutrients and showed the exponential state was maintained for an extra day. By feeding glucose also they have observed that the stationary phase was extended and the death phase delayed they therefore established a simple feeding system to overcome the problem of nutrient limitation.

Mohmad-Saberi *et al*., (2013) looked into different types of growth media which are the RPMI 1640 and Ham's F12 that may affect the culture growth behaviour and productivity. They found out that some similar metabolites were appeared in the different growth medium but with different quantity showing that growth media have effects on the metabolite production. Cells cultured in RPMI media were actively consuming glutamine from early exponential phase which resulted in glutaminolysis activities which correlated to the production of protein of interest and also indicated

the healthiness of cells. From their PCA analysis, asparagine were found to be clustered with IGF-1in the RPMI medium exponential phase, indicating asparagine as a potential signal to the IGF-1 production. From the result they found that a group of sugars (glucitol, talose, glucopyranose, mannopyranose, ribose and fructose) were used as alternatives of glucose in glycolysis. Meanwhile, in death phase, they found lysine, a product of glutamic acid, which is also supplied in the RPMI formulation, suggesting an over-supply of this essential amino acid. Other than that, they also found ornithine which have been reported by other researcher to have apoptotic properties. This study, combining with other data from existing literature, illustrates the usefulness of the metabolomics techniques for improvement of medium formulation by omitting unnecessary nutrients leading to cost reduction or adding other nutrients to avoid or delay limitation.

Another study that looked into culture performance characteristics that correspond to different types of culture medium came from Dietmair *et al*. (2012). CHO cells that were grown in three different types of medium namely CHO-S-SFMII (SFM), HyQ SFM4CHO, (HyQ) and CD-CHO medium showed different growth patterns. Cells in HyQ grew the fastest with the highest cell density which is $7.6x10^6$ cells/mL, and started to decline on day 5. Cell in SFM grow as fast as in HyQ but with only $2.6x10^6$ cells/mL at the highest peak and entered into decline phase on day 6. While cells in CDCHO were maintained until day 7 but with only $3.3x10^6$ cells/mL. Observations from GC-MS showed that an unknown compound was only detected in HyQ culture, which displayed fast growth rates and highest viable cell densities. The intracellular concentration of the unknown decreased during cultivation indicating that the compound was metabolized. From the analyses they found that 14 metabolites

namely dCTP, CTP, ATP, GTP, NAD, UDP-glucuronic acid (UDP-glcA), thymine, glutamine, glutamate, asparagine, histidine, glycine, unknowns 7 and 9 were present in higher concentrations in exponentially growing HyQ and SFM cultures than in exponentially growing CD-CHO cultures. The also did analysis to identify which metabolites were associated with the differences in growth rates of the three culture medium. The results showed only one metabolite being identified which was CTP. Experiments were carried out to examine whether or not CTP which can be generated from cytidine via uridine kinase, affected the slow growth rates in CD-CHO medium. They demonstrated that adding cytidine to CD-CHO cultures did not increase their growth rates. This suggested the difficulties associated with the interpretation of metabolomics data. Future study should be looking into an increasing the coverage of the metabolome and more systematic analysis using computational methods that will facilitate correct interpretation of metabolites analysis.

Carinhas *et al*., (2013) examined the metabolome not only of cultures in different media but also from different clones of cells and at their different growth phases . From the research, they estimated that the higher metabolic efficiency of their CHO cell lines are due to the high asparagine utilization in the nitrogen metabolism and also the used of serine leads to the accumulation of glycine and formate in the culture. The shift from lactate production to consumption increases the metabolic efficiency. However they found that the metabolic differences between cell clones were uncertain.

Instruments used for metabolomics approach specifically in CHO cells culture are nuclear magnetic resonance (NMR) spectroscopy (Bradley *et al*., 2010; Wagstaff *et al*., 2013), near infrared (NIR) spectroscopy (Hakemeyer *et al*., 2012), hyphenated

instruments such as gas chromatography mass spectrometry (GCMS) (Ahn and Antoniewicz, 2011; Mohmad-Saberi *et al*., 2013) and liquid chromatography mass spectrometry (LCMS) (Chong *et al*., 2009; Hounoum *et al*., 2015). There are numerous advantages and also disadvantages of each instrument being used according to the objectives and aim of each experiment. In this study, LC-HRMS will be used as the instruments for metabolite identification.

1.7 Techniques in processing samples for metabolite analysis

There have been a number of studies into the best techniques and procedures for processing samples meant for metabolomics. Steps that are required for sample processing include quenching and extraction of the metabolites from any sample of interest. Sellick *et al.*, (2009) examined the effectiveness of different quenching methods. The quenching solutions used were 60% methanol (MeOH), 60% MeOH with 70 mM HEPES (pH 7.4), 60% MeOH with 0.85% (w/v) ammonium bicarbonate (AMBIC) (pH 7.4) and 60% MeOH with 0.85% (w/v) Sodium Cholride (NaCl). The study focused on measurement of a group of labile metabolites, and found that quenching in -40 °C 60% MeOH supplemented with 0.85% AMBIC was the best method. This is however in contrast with research by Dietmair *et al.* (2010) who compared three different quenching solutions and found the best used for fragile CHO cells sample was cold 0.9% (w/v) NaCl. They also tested cold 60% MeOH (-40 ^{o}C) and 60% MeOH (-40 ^{o}C) buffered with 0.9% (w/v) ammonium bicarbonate (AMBIC, pH 7.4). Both were found to damage the cell membrane and lead to leakage of metabolites.

The next step after quenching is the extraction procedure. As with quenching, there is some disagreement between researchers on the best approach to this. Sellick *et al.,* (2010) tested methanol extraction, methanol/water extraction, hot ethanol extraction, alkali potassium hydroxide (KOH) extraction, perchloric acid (PCA) extraction, methanol/chloroform extraction, methanol/chloroform combined extraction and methanol/chloroform multi-step extraction, and found that the extraction using two 100% methanol extractions followed by a final water extraction recovered the largest range of metabolites. Conversely, when Dietmair *et al*., (2010) looked at 12 different extraction methods, extraction using cold 50% aqueous acetonitrile gave the highest number of intracellular metabolites. In comparison, Volmer *et al*., (2011) believed that it was essential to quickly separate cells from the culture medium because the extracellular component may affect the measurement of intracellular metabolites. Therefore, they proposed a fast filtration method with subsequent extraction of metabolites using cold methanol. Their results were comparable with the other two previous research studies mentioned above and they found no significant leakage of intracellular metabolites using the fast filtration method., Hernandez Bort *et al*., (2014) established an improved protocol for fast filtration, washing and quenching using readily available equipment readily available in any laboratory. They also concluded that drying the sample may cause some loss of metabolites, therefore, they suggested that samples must be directly analysed after extraction process or stored at -80^{o}C. The metabolomics analysis in the present study will be carried out using the protocols and methods of Hernandez Bort *et al*. (2014).

Chapter 2

Materials and methods

2.1 CHO cell lines

The GS-CHO cell-lines, CHO 42 and CHO 124, produce the cB72.3 IgG4 monoclonal antibody with varying levels of productivity were employed in this study. The cell lines were kindly provided by Lonza Biologics (Slough, UK).

2.2 CHO cell culture medium

Three commercially available CHO cell culture media were used in this study, CD-CHO (Life Technologies, Paisley, UK), CD-OPTI CHO (Thermo Fischer Scientific, Grand Island, US) and DYNAMIS (Thermo Fischer Scientific, Grand Island, US).

CD-CHO medium designed for suspension culture of CHO cells and the production of recombinant proteins, in batch and fed-batch cultures. CD-OPTI CHO medium has been further developed from CD-CHO for growth of CHO cells and has been shown to increase product titre in fed-batch cultures. Dynamis medium has been designed to produce the highest cell densities and product titres in both batch and fed-batch cultures of the three media. Dynamis has had the glucose concentration formulated in order to minimise negative effects of lactic acid accumulation.

All media are needed to be supplemented with 1 mL/L of methionine sulfoximine (MSX, Sigma-Aldrich, UK). A stock solution of MSX, (25mM) were first prepared and 1 mL of it will be added into 1 L of respective culture media to get a final concentration of 25 µM of MSX in the complete culture medium. This is because the

cell lines used in this study were GS-CHO cell lines where this Glutamine Synthetase (GS) system can be grown in medium without glutamine. GS is the enzyme that responsible for the biosynthesis of glutamine from glutamate and ammonia and is very important for the survival of mammalian cells in culture without glutamine. However, CHO cell lines express sufficient GS to survive without exogenous glutamine, therefore, MSX was added as the GS inhibitor to inhibit endogenous GS activity. The medium will be mixed by inversion. Prior to use, the complete medium was warmed to 37^{o}C in a water bath for 30 minutes.

2.3 Thawing of original cell bank vial

30 mL of complete medium were transferred to a 125 mL Erlenmeyer flask with vent cap (VWR International, Leicestershire, UK). A culture vial was removed from the liquid nitrogen storage tank and located in the -80^{o}C freezer overnight. The vial was transferred in a freezing container. The vial was sanitized with 70% v/v isopropyl alcohol (IPA) (Sigma Aldrich, UK) before being transferred to the 37^{o}C water bath for thawing. As soon as there was only a small fragment of ice in the tube, the vial was transferred to the biological safety cabinet and again the vial was sanitized by spraying with 70% v/v IPA. A sterile Pasteur pipette was used to transfer the contents of the vial drop-wise into a 125 mL Erlenmeyer flask containing a suitable medium accordingly (CD-CHO, CD-OPTICHO or Dynamis). Flasks were incubated in the 37^{o}C, 5 % CO_2 incubator overnight and utilised immediately the following day.

2.4 Monitoring the growth of GS-CHO cells

Following vial-thaw the cell count and viability of cells were determined by taking a 1 mL sample from the flask into an Eppendorf tube using a 1 mL sterile pipette in the biological safety cabinet. The flask was placed back in the 37^{o}C, 5 % CO_2 incubator overnight. Cell concentration and viability were determined with a haemocytometer (Improved Neubauer, Hawksley, West Sussex, UK). The trypan blue (Biochrom, UK) exclusion method was used to distinguish between viable and non-viable cells. The cover slip and haemocytometer used were cleaned with alcohol and lint-free tissue paper. The cleaned cover slip was fixed to the haemocytometer. Figure 2.1 shows the appearance of the haemocytometer with the application of sample and the haemocytometer grid visualized under the microscope.

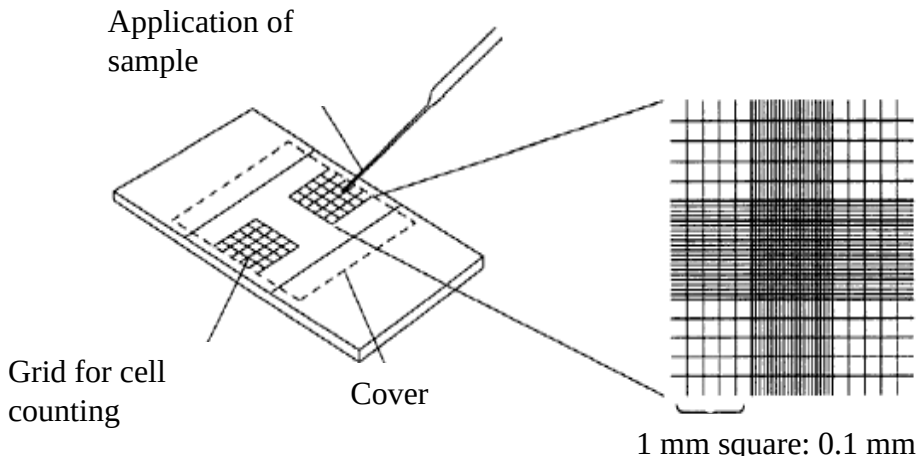


Figure 2.1 Appearance of the haemocytometer grid visualized under the microscope (Butler, 2004).

For a 10x dilution, 10 µl of sample was mix with 90 µl of trypan blue solution. The mixture was re-suspended vigorously using a pipette and vortexes. After that, 10 µl of the mixture was pipetted at the edge of the cover slip and allowed to flow under it. The haemocytometer was visualized under an inverted microscope. Trypan blue will penetrate the cell wall of a dead cell; therefore, dead cells will appear blue under the microscope while live cells will appear bright and colourless. Viable (live) and dead cell number was calculated from the average of the total four large squares in the haemocytometer.

The calculation for viable (live) and dead cell number is shown below:

Cells/ mL = the average count per large square X dilution factor X 10^4
(conversion factor of the heamocytometer)

Dilution factor = $\frac{\text{[volume of sample + volume of diluent (trypan blue)]}}{\text{volume of sample}}$

2.5 Feeding the GS-CHO cells

Complete CD-CHO medium was pre-warmed in a water bath set at 37ºC for 30 minutes before inoculation. Cell count and viability of cells were determined. Cells were transferred into a 50 mL sterile tube and cells were harvested by centrifugation for 5 minutes at 1100 rpm. The supernatant was discarded by decanting into a waste bottle and the cell pellet was re-suspended in 30 mL of fresh medium. The contents were swirled gently to mix and were transferred into a sterile 125 mL Erlenmeyer flask with vent cap. The flask was placed in the 37ºC, 5 % CO_2 incubator overnight. The viability of cells was determined every 24 hours after resuscitation.

2.6 Sub culturing/ Passaging GS-CHO cells

The cells are ready to be split once they reach a cell density of ~$1x10^6$ cells/mL, with >90 % viability achieved. Complete medium (2.2) was pre-warmed for 30 minutes in a water bath set at 37ºC prior to cell transfer. Cell count and viability of cells were determined. 27 mL of pre-warmed complete medium were transferred into 125 mL flask. 3 mL of culture from the previous flask (2.5) were taken and inoculated into the 125 mL flask. The flask was then used as the seed culture flask for subsequent use in transient transfections. Figure 2.2 shows the work flow for sub culturing/passaging GS-CHO cell lines and the preparation of working cell bank for different passage number of cells.

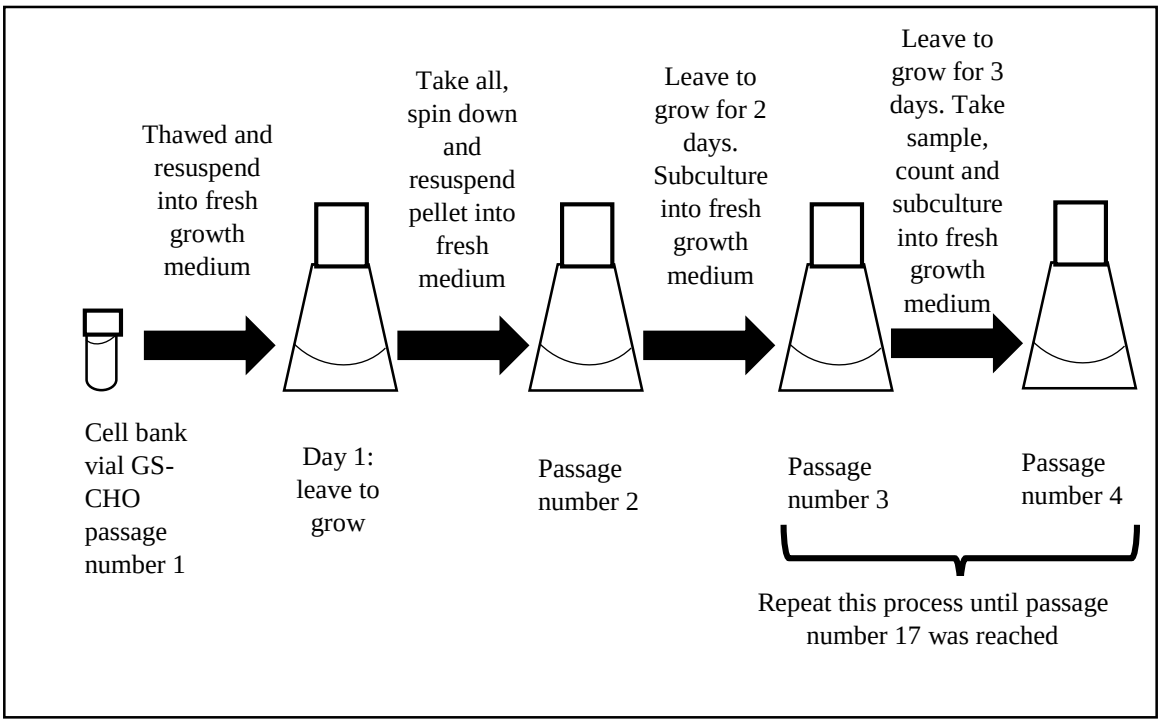


Figure 2.2 Work flow for sub culturing/passaging GS-CHO cell lines and the preparation of working cell bank for different passage number of cells.

2.7 Cryopreservation

The remaining cell suspension from the previous flask (2.6) were harvested by centrifugation for 5 minutes at 1100 rpm. The spent medium was discarded and volume of cell freezing medium (complete medium + 7.5 % v/v of DMSO) required to achieve a final cell density of $1x10^7$ cells/mL was calculated using the worked example as below:

Worked example

Total cell count = $1.18x10^6$ cells/mL x 27 mL (cell suspension volume)

Total cells = $3.19x10^7$ cells/mL

Therefore,

$3.19x10^7$ / $1x10^7$ = 3 mL cell freezing serum-free medium DMSO was added to the cell pellet.

This would provide 3 cell bank vials with 1 mL per vial. Vials were labelled appropriately. Vials were then transferred to a freezing container and were placed in the -80 ^oC freezer overnight. The next day the vials were transferred to the liquid nitrogen storage vessel.

2.8 Calculations

Cell growth, specific growth rate, doubling time and generation number were calculated using the following equations. Specific growth rate (μ):

Specific growth rate (μ): $$\mu = \frac{ln\frac{X1}{X0}}{t1-t0}$$

Doubling time (dt): $$dt = \frac{ln(2)}{\mu}$$

Where:

t_0 and t1 is the time at points 0 and 1, respectively

X_0 and X_1 is the viable cell density at time points 0 and 1, respectively.

2.9 Shake flask culture

Shake flask culture was used in the experiments discussed in Chapter 3; Characterisation of the physiology of the industrial CHO cell lines in shake flask culture was carried out using three biological replicates of 125 mL disposable shake flask with vent cap (VWR International, Leicestershire, UK) with a working volume of 30 mL. Cells were incubated at 37^{o}C and 5% CO_2 on an orbital shaker (Minitron, Infors HT, Bottmingen, Switzerland) at 150 rpm.

GS-CHO cell line used for both different passage studies and different cell types studies, (GS-CHO 42 or GS-CHO 124) were taken from the working cell bank to be revived and prepared as mentioned in section 2.3, 2.4, 2.5 and 2.6. The summary for the process are shown in Figure 2.3 (Example below is for preparing the seed culture for GS-CHO 42 passage number 4; however the process were applied to all passage number of cell that need to be prepare).

Figure 2.4 (A) shows three biological replicates of shake flask ready incubated in the incubator (Fig. 2.4 B).

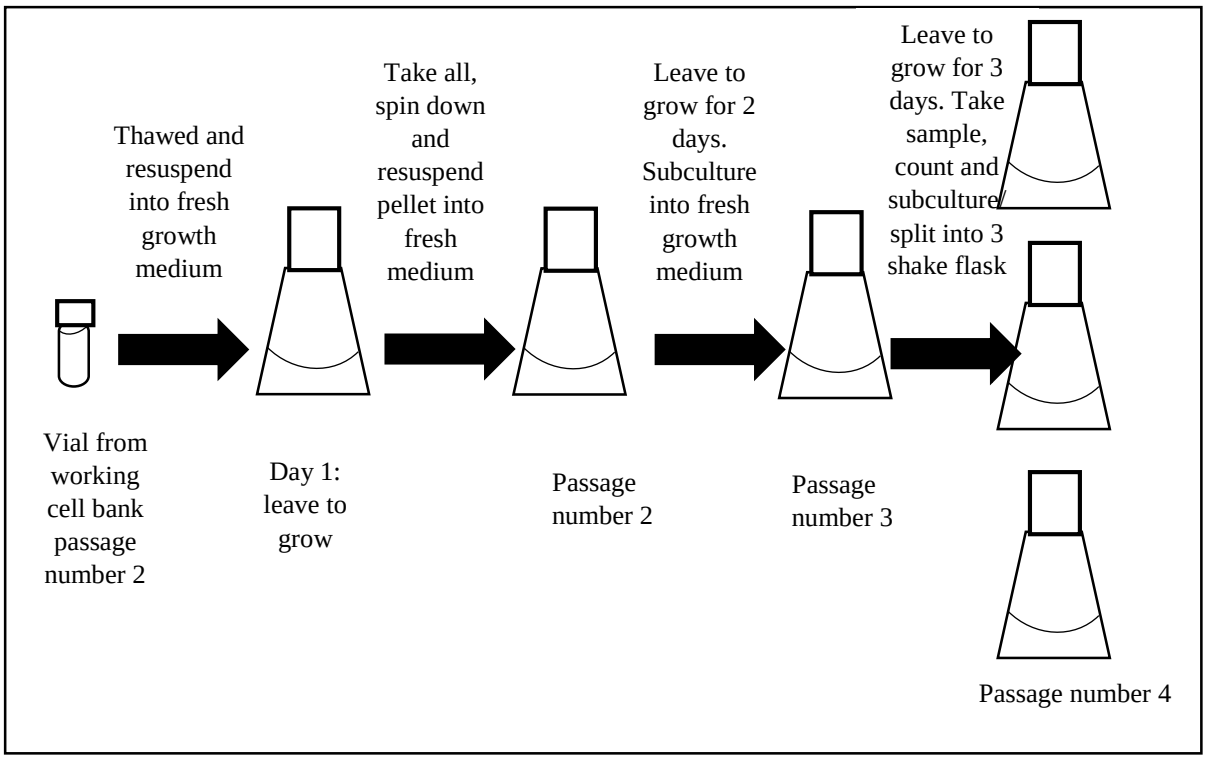
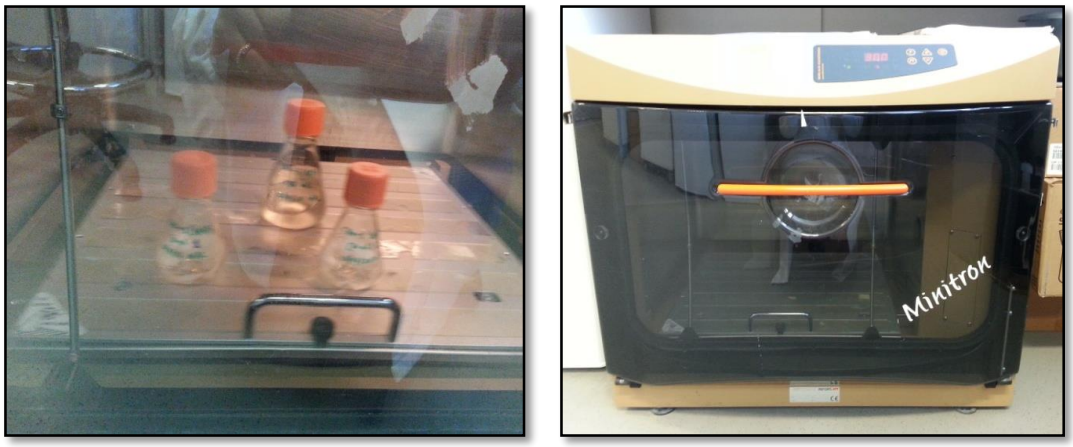


Figure 2.3 Work flow for the preparation of seed culture for the study in shake flask.



A B

Figure 2.4 A) Three biological replicates of shake flask ready incubated in the incubator. B) Incubator shaker used in this study (Minitron, Infors HT, Bottmingen, Switzerland).

2.10 Batch cultures

This method is pertinent to the experiments in Chapter 4.

Characterisation of the physiology of the industrial CHO cell line in bioreactor batch culture was carried out in two 5 L bioreactors (*Applikon* Biotechnology BV, The Netherlands) and were controlled by a Bio Controller ADI 1010 and by a *Bio Console* ADI 1025 (*Applikon* Biotechnology BV, The Netherlands) running parallel with a starting working volume of 3 L.

The bioreactors consisted of borosilicate glass vessel supported with stainless steel scaffold and attached to a stainless steel head plate (Fig. 2.6 B). The head plate accommodates the on-line probes for temperature, pH and dissolved oxygen. The operating parameters (temperature, pH, dissolved oxygen) in the bioreactors were set in a Bio controller ADI 1010 located next to the bioreactors. Agitation was provided by a Rushton turbine impeller operating with an agitation rate of 250 rpm. Dissolved oxygen concentration was kept at 40% of saturation by sparging with air and pure oxygen gas via an open pipe sparger at a flow rate of 100 mL/min. Temperature is controlled at 37^{o}C by an electrical heating blanket covered around the glass vessel. Culture pH was kept constant at 7 by addition of 0.5 mM of sodium carbonate buffer (NaOH) or by sparging CO_2. The vessels were sterilized at 121^{o}C for 20 minutes, with 2 L of water, which was removed aseptically after sterilization. Cells were seeded at a density of 0.3 x 10^6 cells/mL. The CD-CHO medium (Life Technologies, Paisley, UK)

was supplemented with a 1% (w/v) solution of antifoam C emulsion (Sigma Aldrich, UK) and 25μM of methionine sulphoximine (MSX) (Sigma Aldrich, UK) was transferred aseptically through the prepared media line. Seed for the bioreactor batch culture were prepared initially as described in the schematic diagram shown in Figure 2.5.

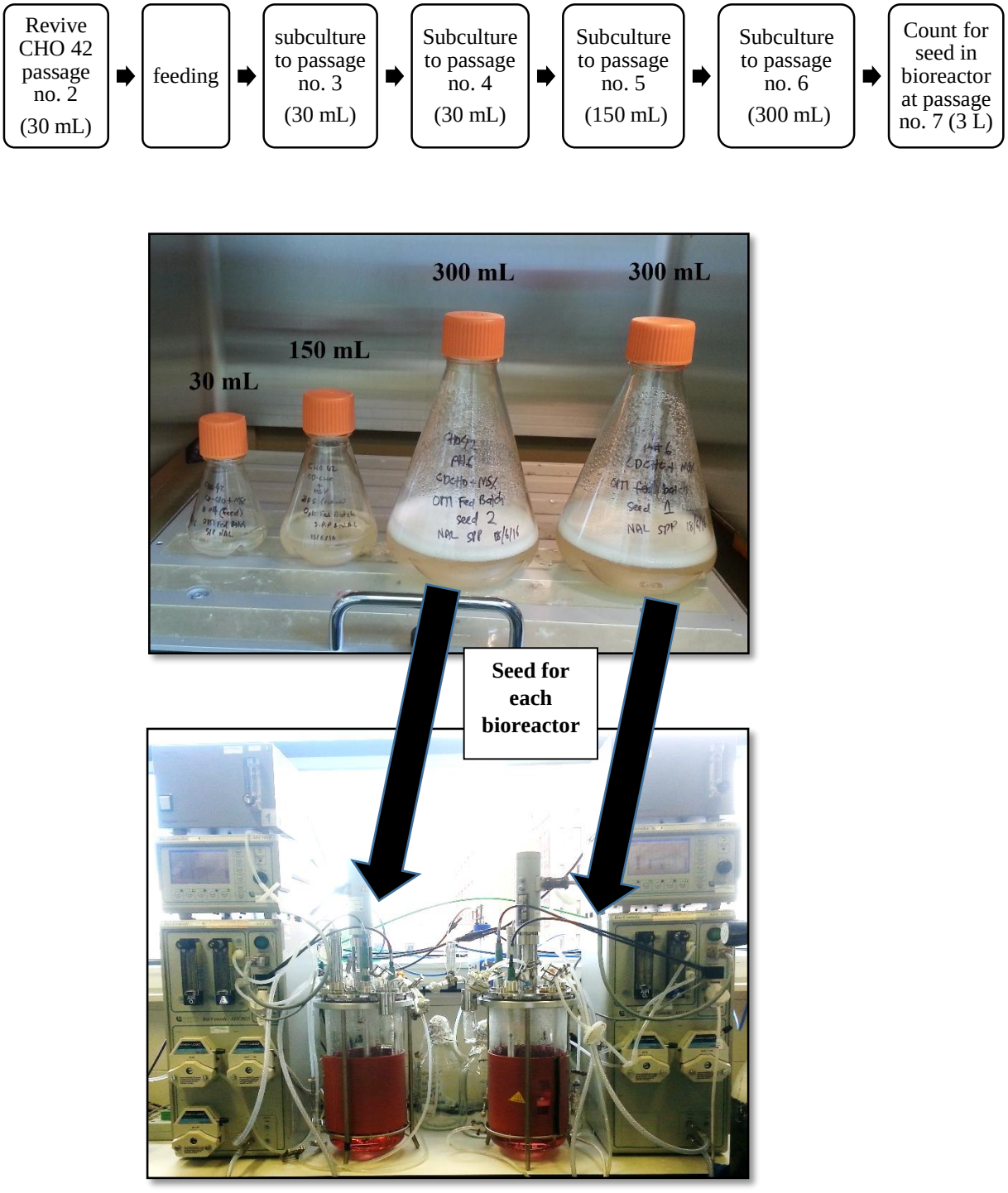


Figure 2.5 Schematic diagram for the preparation of seed culture for the bioreactor batch culture.

2.11 Fed Batch cultures

The seed for fed batch CHO cell culture were prepared initially, accordingly to the batch culture protocols (Section 2.10). The feeding strategy applied in this experiment was referred to a research study by Barrett *et al*., (2012). The bioreactor culture was fed with 5.6% CHO CD Efficient Feed B (EFB) feeding supplement for study using CD-CHO media on days 3, 5, 7, 9, 11, 13, and 15 (40% total). While for study using CD-OPTICHO media, the bioreactor culture was fed with 10% CHO CD Efficient Feed A (EFA) feeding supplement on days 4, 6, and 8 (30% total). The bioreactor parameter were as the same as batch culture (section 2.10).

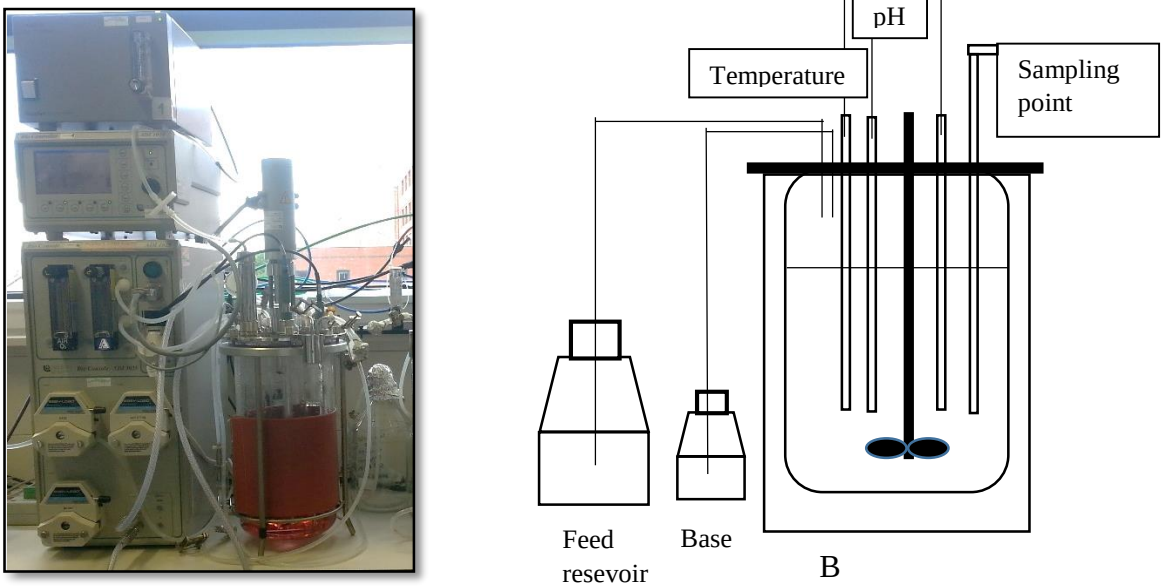


Figure 2.6 A) 5 L bioreactors controlled by a Bio Controller ADI 1010 and by a *Bio Console* ADI 1025 (*Applikon* Biotechnology BV, The Netherlands). B) Schematic diagram of bioreactor set up.

2.12 Analytical methods

At a fixed time interval (daily), one mL of cell culture was taken from each flask for cell concentration and viability analysis. Samples were first centrifuged to remove the cells and the supernatant was stored at -20^{o}C prior to analyse**.**

2.12.1 Quantification of Glucose, Lactate, Glutamate and Ammonia

Glucose, lactate, glutamine and ammonia were all measured using a Biochemistry Analyser, YSI 2950 (YSI Incorporated, Hampshire, UK). The YSI analyser used an enzyme sensor technology, where each enzyme probe consisted of three layers; cellulose acetate membrane, immobilized enzyme and polycarbonate membrane (Figure 2.7). Samples containing substrate diffuse through the membrane and reaction 1 occurs when the analyte contacts the immobilized oxidase enzyme. This is where the substrate will be rapidly oxidized producing hydrogen peroxide (glucose is used as an example). The hydrogen peroxide (H_2O_2) is then oxidized at the platinum anode and produces electrons (Reaction 2). A steady state response will occur when the amount of H_2O_2 produced during reaction 1 is equal to the amount of H_2O_2 that leaves the immobilized enzyme layer. The concentration of substrate is determined from the electron flow that is linearly proportional to the steady state concentration of H_2O_2.

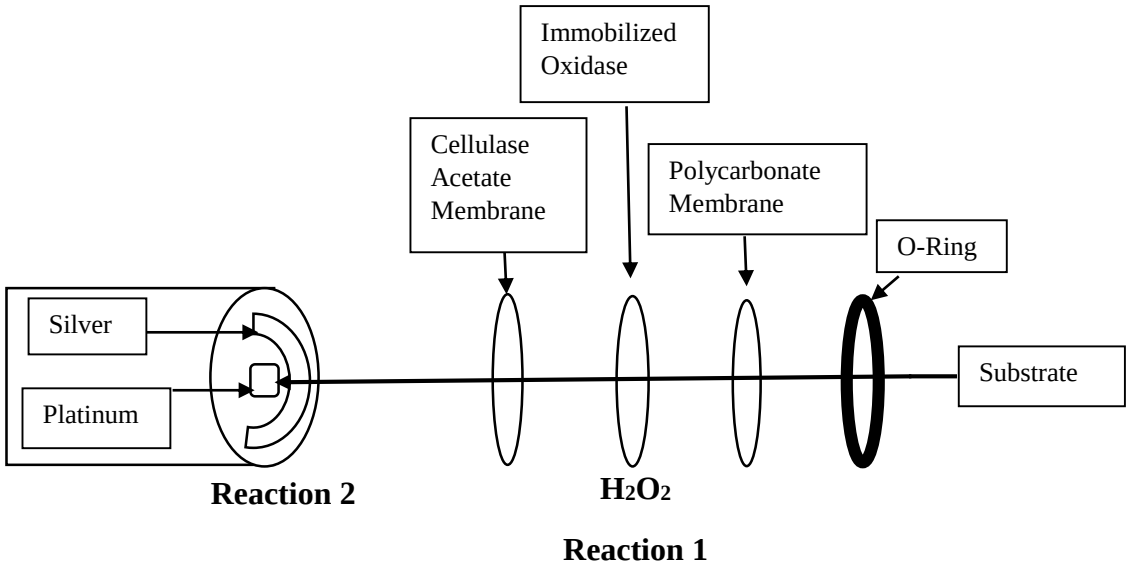


Figure 2.7 Sensor probe and enzyme membrane. (Diagram adapted from the YSI2950 user manual).

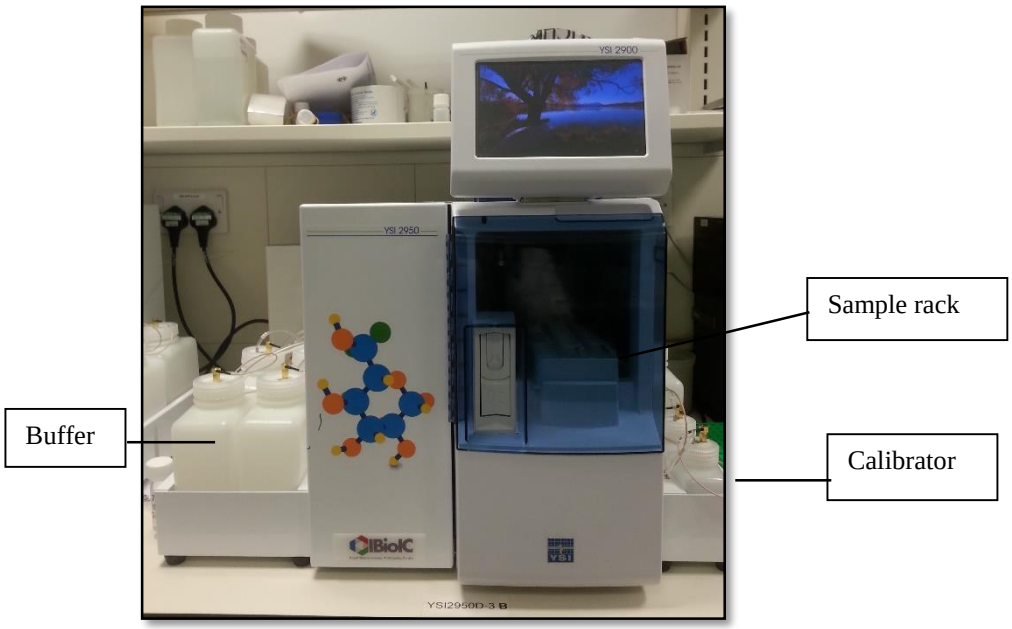


Figure 2.8 YSI 2950D Biochemistry Analyzer

2.12.2 Enzyme-linked immunosorbant assay for immunoglobulin G (IgG) quantification

A human IgG ELISA kit (Bethyl Laboratories, Universal Biologicals, Cambridge, UK) was used to determine the IgG concentration in culture fluids. The kit consists of Human IgG Pre-coated 96 well strip plate, Human IgG standard, Human IgG Detection Antibody, 10X Dilution buffer B, HRP Solution A, TMB substrate, Stop solution, 20X wash buffer and sealing tape. The kit is based on a sandwich ELISA where IgG present in sample will be captured by anti-human IgG antibody that has been pre-adsorbed on the surface of microtiter wells. Before inoculating sample into the 96 well plate, the samples were diluted into 1:10000. 10 µl of sample was added to each well including the standard and controls. The plate was covered and incubated at room temperature for 1 hour. Then, the plate was washed four times using the washing buffer (200 µl per well). 100 µl of anti-hIgG detection antibody were added to each well, covered and incubated at room temperature for 1 hour. The plate was washed four times and 100 µl of HRP solution A were added to each well. After that, the plate was covered and incubated at room temperature for 30 minutes. Then the plate was washed four times as before. 100 µl of TMB substrate solution were added to each well and the plate was incubated in the dark at room temperature for 30 minutes. After 30 minutes, the reaction was stopped by adding 100 µl of stop solution to each well. The absorbance in each well was measured on a plate reader at 450nm.

2.13 Metabolomics and metabolite analysis

2.13.1 Quenching and extraction of metabolites

Metabolite sampling is generally a two steps process consisting of quenching to stop metabolism and allow medium removal, followed by extraction to recover the metabolites from the cells. It is most important for metabolite sampling to stop the organism's metabolic activity quantitatively and rapidly. This is to avoid loss of metabolites caused by residual enzyme activity or quenching conditions. There have been a number of studies to look at the best techniques and procedure for processing samples meant for metabolomics. Steps that are required for sample processing include quenching and extraction of the metabolites from any sample of interest. Sellick *et al.,* (2009) examined the effectiveness of different quenching methods. The quenching solutions used were 60% MeOH, 60% MeOH with 70 mM HEPES (pH 7.4), 60% MeOH with 0.85% (w/v) ammonium bicarbonate (AMBIC) (pH 7.4) and 60% MeOH with 0.85% (w/v) Sodium Chloride (NaCl). The study focused on measurement of a group of labile metabolites, and found that quenching in -40 °C 60% MeOH supplemented with 0.85% AMBIC was the best method. This is however in contrast with research by Dietmair *et al.* (2010) who compared three different quenching solutions and found the best used for fragile CHO cells sample was cold 0.9% (w/v) (NaCl). They also tested cold 60% MeOH (-40 ^{o}C) and 60% MeOH (-40 ^{o}C) buffered with 0.9% (w/v) AMBIC, (pH 7.4). Both were found to damage the cell membrane and lead to leakage of metabolites. Therefore, alternative methods for the quenching of suspended animal cells are required. It is very important to separate the cells from

supernatant before metabolite extraction, thus making filtration by centrifugation the most suitable techniques. Volmer *et al.*, (2011) describe new method for sampling suspended animal cells using fast filtration and subsequent extraction of metabolites. They found out that the combination of fast filtration and cold methanol extraction is suitable for intracellular metabolomics studies of suspended animal cell cultures and better to other methods currently applied due to no leakage of metabolites and ensured rapid and efficient quenching of samples before extraction. Hernandez Bort *et al*., (2014) developed a modified protocol for fast filtration, washing and quenching using readily available parts. Their study on reduced quenching and extraction time for mammalian cells using filtration and syringe extraction showed that the speed of their method would be of advantage because the procedure are easy and fast to enable generation of a high number of sample and the equipment needed are easily found in any laboratory. Culture fluid was collected and processed according to the method of Hernandez Bort *et al*., (2014). The filtration process used a Buchner funnel filter set where 1 x 10^7 cells from the culture were collected and filtered through a humidified filter membrane (PALL A/D Glass Fibre, 47, Fisher Scientific, UK). The filter membrane humidification was achieved using 10 mL of 0.9% (w/w) NaCl. Then, cells were immediately washed with 30 mL of 0.9% (w/w) NaCl solution to remove extracellular contaminating substances. After that, the filter membrane with the captured cells was transferred into a 20 mL syringe barrel (20 ml BDTM Syringe with Luer-LockTM connection, Sigma, UK) closed with a cap and snap freeze in liquid nitrogen. Extraction were carried out using 4 mL of cold methanol added into the syringe barrel. Then the plunger was carefully reinserted into the syringe barrel after removing the Luer-LockTM cap and slightly pushing on the plunger to remove the

excess air inside. The syringe barrel was re-locked with a Luer- LockTM cap and vortexed for 15 s. The cap was removed and a 0.45 µm filter membrane unit (Millex HV-Durapore PVDF filter, Millipore, UK) was attached to the syringe. The syringe plunger was pressed to transfer the extraction solution into a fresh tube. The entire extracted volume was stored at −80°C for further quantification of metabolites. Figure 2.9 shows the flow chart of quenching and extraction technique for metabolite sample preparation.

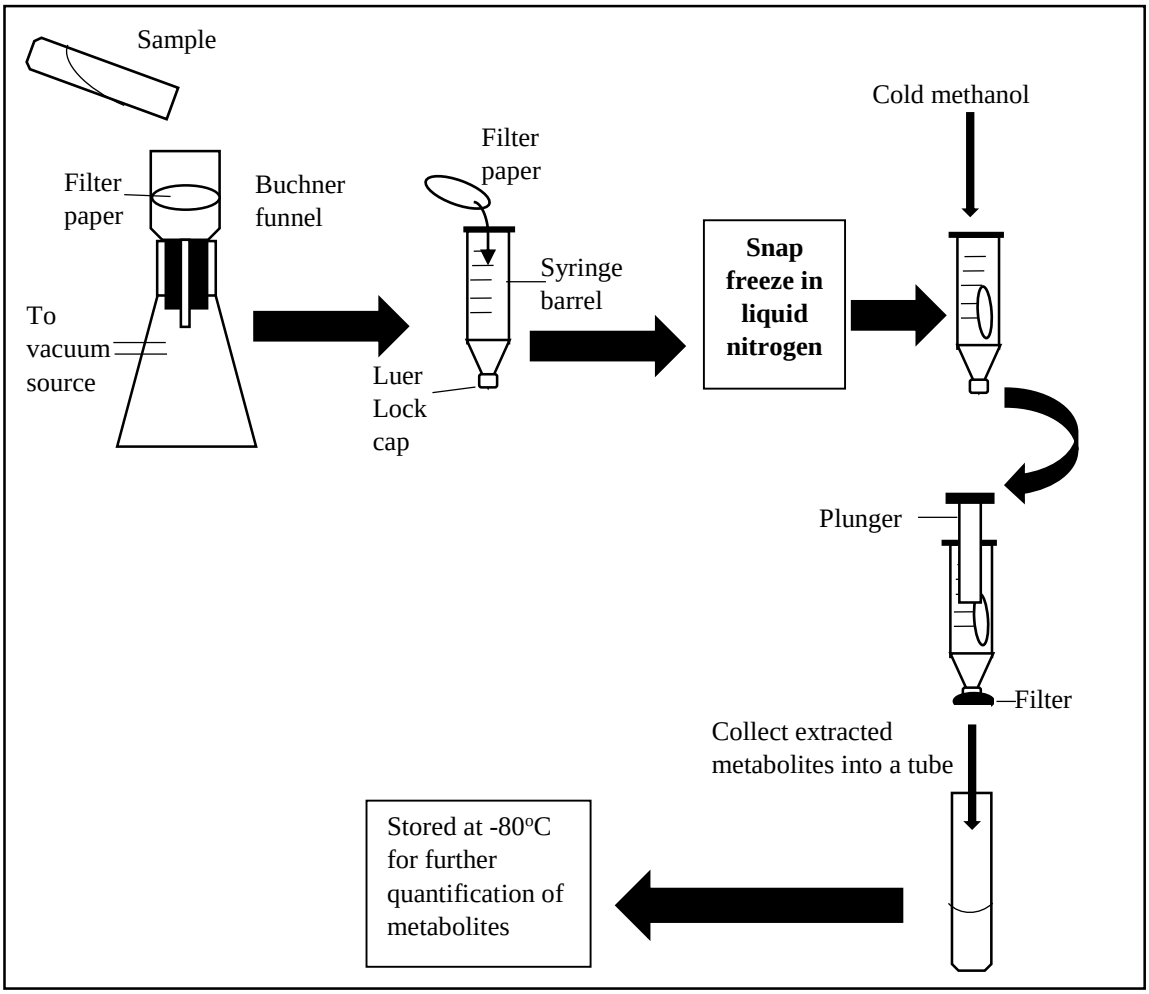


Figure 2.9 Flow chart of quenching and extraction technique for metabolite sample preparation**.** (Adapted from Hernandez *et al*., 2014).

2.13.2 Liquid chromatography- high resolution mass spectrometry (LC-HRMS) analysis

10µl of extracted samples were injected into an ACE 5 C18 75x3.0 mm (Hichrom Ltd, UK) maintained at 20ºC. The solvents used were A: 0.1% formic acid (Fisher Scientific) in deionized MilliQ water and B: 0.1% formic acid (Fisher Scientific) in acetonitrile (HPLC grade, Fisher Scientific). A 45 minute gradient was employed (10-100% B 30 min, 100% B 5 min, 100-10% B 1 min, 10% B 10 min) at a flow rate of 300 µl/min. The LC eluent was directed to an Exactive Orbitrap MS (Thermo Scientific). Electron spray ionization was carried out in both positive and negative modes in full scan with the Orbitrap for masses between m/z 150-1500 at 30,000 resolution and three microscans. Sheath gas was set at 50 (arbitrary units), aux gas at 17 (arbitrary units) and the capillary temperature was at 320 °C. The capillary voltage and electrospray voltage were 32.5 V and 4.5 kV respectively for positive mode ionization, and -25 V and 4.0 kV respectively for negative mode ionization.

2.13.3 MS data processing

The raw MS data were pre-processed using Xcalibur software and converted into positive and negative sliced data separately. These data were then further processed in MZmine software. A list of masses were generated with mass detection using centroid mass detector where it assumes that each signal above the given noise level (1000) is a detected ion. After that, the chromatograms were built. Chromatogram builder takes mass lists generated for each MS scan and builds a chromatogram for each mass that can be detected continuously over the scans. Later, the chromatogram will be de-convulated into individuals' peaks by using the local minimum search where it searches for local minima in the chromatogram and separates individual peaks at these minimal points. The minimum relative height was set at 5% and peak duration range at 0.2-5 min. After that the process continues with the isotopic peak grouper where peaks in a peak list which form an isotope pattern were found. Additional isotopic peaks were removed from the peak list and only the highest isotope was kept. Then, an alignment was used to align detected peaks in different samples through a match score. For a given peak from one experiment, the algorithm attempted to find the closest matching mass peak in terms of mass and elution time within a pre-defined threshold in other experiments. After alignment, some missing peaks that were not identified by the algorithm will be filled in using the gap filling peak finder method. The m/z tolerance was set at 0.001 m/z or 5 ppm and the RT tolerance was set at 0.5 min. After gap filling, all peaks found in solvent blanks and peaks with intensity more than $5x10^4$ were deleted. Then, identification of peaks was carried out using the online databases, Kyoto Encyclopedia of Genes and Genome (KEGG) and Human Metabolome Database (HMDB). Positive and negative data obtained from MZmine

were exported as a CSV file for further processing using an in house macro Excel, where it combined the positive and negative ionization for further analysis. Later, another CSV file was created to be operated for statistical analysis in SIMCA-P V14.0 (Umetrics, Umea, Sweden). An unsupervised principal component analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were used to detect different features found in the samples. Analysis using SIMCA-P+ version 14.0 was also used to identify any putative metabolite in the loading plot and the peak intensity of the metabolite was investigated across the sample set.

Chapter 3

Characterisation of the physiology of industrial CHO cell lines in shake flask batch cultures

3.1 Introduction

Production of biopharmaceuticals needs improvement and research into the upscale process where the selection of the best of cell lines is made, and the best clones picked for further process development. The study of choosing the best conditions for cell growth with high density and productivity will have a very high cost if they were done at scale, for example in the bioreactor. Therefore, studies at a small scale, for example, in shake flask that will resemble the production of antibody in a larger scale will help keep down the cost of the study. Shake flask use is a resource intensive approach, but is being used to generate large numbers of experiments with a small volume of medium being used leading to more cost effective research. Lonza Biologics have kindly provided two types of GS-CHO cell lines for this research study, which are a high producer (GS-CHO 42) and low producer (GS-CHO 124) cell lines. The present studies were done in order to choose which cell line gave better results in term of robust, fast growth and high-production. The first part of this chapter will be focusing on the different types of cell lines, which both expressed recombinant chimeric IgG4 mAb.

In the second part of this chapter, the effect of different cell passage number and different type of commercially available culture medium on cell growth performance were examined. Using the selected industrial cell line, various commercial media supplied by another industrial partner (Thermo Life Sciences) were used for production of antibody. According to Oltra-Noguera *et al*., (2015) in their study of evaluation of the effect of passage number on the permeability values and cell culture

transport experiments, passage number of cells used in their experiment were between 10 to 80 post-defrosting cell lines and it showed that lower passage number gave better results. The normal passage number that were used by several researchers in their study were below 10 (Fernandez Martell, 2015; Reinhart *et al*., 2015); this is because lower passage number gave better results in term of cell density and productivity. In the present study, passage number 17 were used because we consider it as a high passage number after sub culturing 18 times. GS-CHO 42 were sub-cultured several times until they reached passage number of 17 to be used in this study. The time frame for getting to cell line passage number 17 was illustrated in Chapter 2 (Figure 2.2).

In this Chapter, the objective is to look whether high passage number give any significant effect to the cell growth performance. Based on the findings by Heinrich *et al.*, (2011) cultivations of four different stages of sub-populations of CHO cells showed differences in specific growth rates and product formations and also differences in the metabolism of glucose, lactate and some other amino acids. Similar to that, Beckmann *et al.*, (2012) detected growth performance and intracellular adenylate energy charge increased during cultivation of high passage cell lines. They also observed that productivity of cells increased at the beginning but decreased at a higher passage cultivation. From the four subpopulations that they examined, the highest passage subpopulation gave a below limit of detection (LOD) for the product concentration in all performed cultivation. While the other three lower passage have a reading between 7.5 to 10.5 pg/cell per day of specific antibody production.

There are also some research groups using non-CHO cells studying passage numbers effects. Canine adipose-derived mesenchymal stem cells (cAD-MSCs) were found to demonstrate changes in their proliferation ability and multipotency after serial sub-passaging (Lee *et al.*, 2014).While Erac *et al.*, (2014) in their study using embryonic rat aortic smooth muscle cells (A7r5) showed that growth rate and intracellular Ca^{2+} dynamics were altered during sub-culturing and suggested that passage number and the sub-culturing process should be clearly stated for a precise evaluation of published data. Another study using Madin-Darby canine kidney (MDCK) cells showed that there was an appropriate passage number that optimized oligosaccharide production and that higher passage number resulted in a decrease in oligosaccharide production (Shimura *et al.*, 2012). Although it had been showed from several research that lower passage number gave better results for cell density and productivity, it is still crucial to investigate the behaviour of the GSCHO cell lines used in this study whether using higher passage number of cell gave any significant different results comparing to using the lower passage number. Results can also be used to look at what passage number the cell lines should be stop from sub culturing; if any divergent effects of long term culturing on cell culture physiology being monitored.

In the present study both passage number 4 and passage number 17 will be cultured in two types of medium which are the CDCHO and CDOPTI medium which were kindly provided by ThermoFisher Scientific, one of the leading biopharmaceuticals company. As Lonza provided the cells, they were already successfully adapted to grow in CD-CHO media. However, according to ThermoFisher, CD-OPTICHO medium is specifically designed to offer high performance and yield that is capable of gaining 2-5 fold increases in fed-batch over batch culture. Reinhart *et al.*, (2015) studied eight

commercially available, chemically defined CHO cell culture media from different companies. They argued that the lack of published data examining the effect of using different cell culture media on cell metabolism and antibody production, clearly indicated a need to test and evaluate the different media from different vendors. They found that existence of even small metabolites might be a good indicator to look at the performance of cell growth. This is because cells cultured in two of their tested medium which are the CD-CHO and FortiCHO did not grow to high concentration, although a constant growth rate were shown. This condition suggested that cells may undergo apoptosis that released DNA from dead and decaying cells and leads to the formation of cell clusters or aggregates which with even in a small amount might be an indicator of the performance of the cell growth. In addition,some media had higher glucose concentrations than others and this may have induced the suppression of respiration and oxidative phosphorylation and led to higher lactate production. Dietmair *et al.*, (2012) observed the metabolite profile of CHO cells with different growth characteristics. They tested and adapted CHO cells in three different commercial media, CHO-S-SFMII, HyQ SFM4CHO and CD-CHO and using a metabolomics approach observed metabolites present in the culture that may affect cell growth performance and productivity in different culture media. Other researchers also examined media formulation by adding a selected component to the commercially available media in order to meet the requirement of cell growth performance and productivity of antibody. For example, Hazeltine *et al.*, (2015) tried to lessen the tryptophan oxidation in a chemically defined medium by increasing tryptophan levels, copper and manganese and decreasing cysteine concentrations. Substantial increase of tryptophan oxidation in the complimentary determining region (CDR) where CDR

plays important role in antigen binding may lead to devastating impacts from immunogenicity to loss of efficacy to the end product (Hazeltine *et al*., 2015). According to them, addition of one or two oxygen atoms to the indole ring of its side chain and other subsequent degradation end products were caused by the oxidation of tryptophan. However, by the suggested procedure of lowering trytophan oxidation in the media and feed, acceptable product quality profile and high titer were maintained. Also Hossler *et al.*, (2015) stated that by adding bioflavonoid chemical family into cell culture media may reduce acidic species charge variants in recombinant proteins that may affect protein stability, activity, immunogenicity and pharmacokinetics.

3.2 Methods

The cell lines were grown in batch cultures in three replicates. Culture were in 125 mL shake flasks with a working volume of 30 mL in CD-CHO medium + MSX. The cell numbers were determined by the trypan blue exclusion methods using a haemocytometer (Section 2.4) and the antibody concentrations were determined using ELISA (Section 2.10.2), routinely throughout the culture duration until the cells reached death phase. Daily samples of 1 mL also were kept for extracellular metabolites quantification (Section 2.10.1).

3.3 Results and discussions

3.3.1 Characterisation of different types of GS-CHO cell lines; a high and low producer cell lines.

In batch experiments of the shake flask,, the impact of the different cell types on cell growth and antibody concentrations, as well as the process duration, was evaluated (Fig. 3.1 (A), (B)). A summary of the data generated from the batch cultures is given in Table 3.1.

Table 3.1 Process relevant data from batch culture in shake flask for GS-CHO 42 (high producer) and GS-CHO 124 (low producer)

Cell Type	**GS-CHO 42**	**GS-CHO 124**
Peak cell conc. (10^6 cell/mL)	7.87	8.35
Antibody conc. (g/L)	0.17	0.15
Specific growth rate, μ (h^{-1})	0.032	0.033
Doubling time (h)	21.66	21.00

The growth for both GS-CHO cell lines were not significantly different (P-values>0.05) as computed using T-test analysis. However, there is a slightly higher cell density from GS-CHO 124 which is the lower producer. GS-CHO 124 cells grow up to 8.35 x 10^6 cells/mL on day 7 compared to GS-CHO 42 which grow up to 7.87 x 10^6 cells/mL. A study by Carinhas *et al.*, 2013 showed a similar pattern where a low producer clone gave higher viable cell numbers and also exhibited a prolonged stationary phase in culture compared to a high producer clones. In the present study, the viability for both cell lines were maintained above 95% throughout the culture and only decreased after day 7 going into their death phase. In the present study, the IgG concentration reached of 0.17 g/L of IgG on day 9 for the higher producer cell line and 0.15 g/L IgG from the lower producer. However the differences were not significant as tested by the T-test. The IgG concentrations were considered low compared to the amount given in previous literature which also culture CHO cell line in shake flask (Reinhart *et al*., 2015 and Nienow *et al*., 2013). According to the research by Nienow *et al*., (2013), pH was maintained at pH 6.9 within the culture process for a small scale bioreactor vessel and 5 L bioreactors. They also did an offline analysis for shake flask culture which displayed a much larger pH variation ranging from 7.4 at the start to pH 6.7 during the rapid growth phase. While for dissolved oxygen, both bioreactor systems, (small scale and 5 L) were controlled at 50% saturation, however in shake flask, the values fluctuated, making it impractical to be measured. These limitations of shake flask culture (oxygen transfer and pH control) may be the main reasons of the low concentration of IgG amount obtained from the shake cultures in the present studies. Table 3.1 shows the specific growth rate of both cell lines. GS-CHO 124 have a slightly higher growth rate which is 0.033 h^{-1} compare to 0.032 h^{-1} for GS-CHO

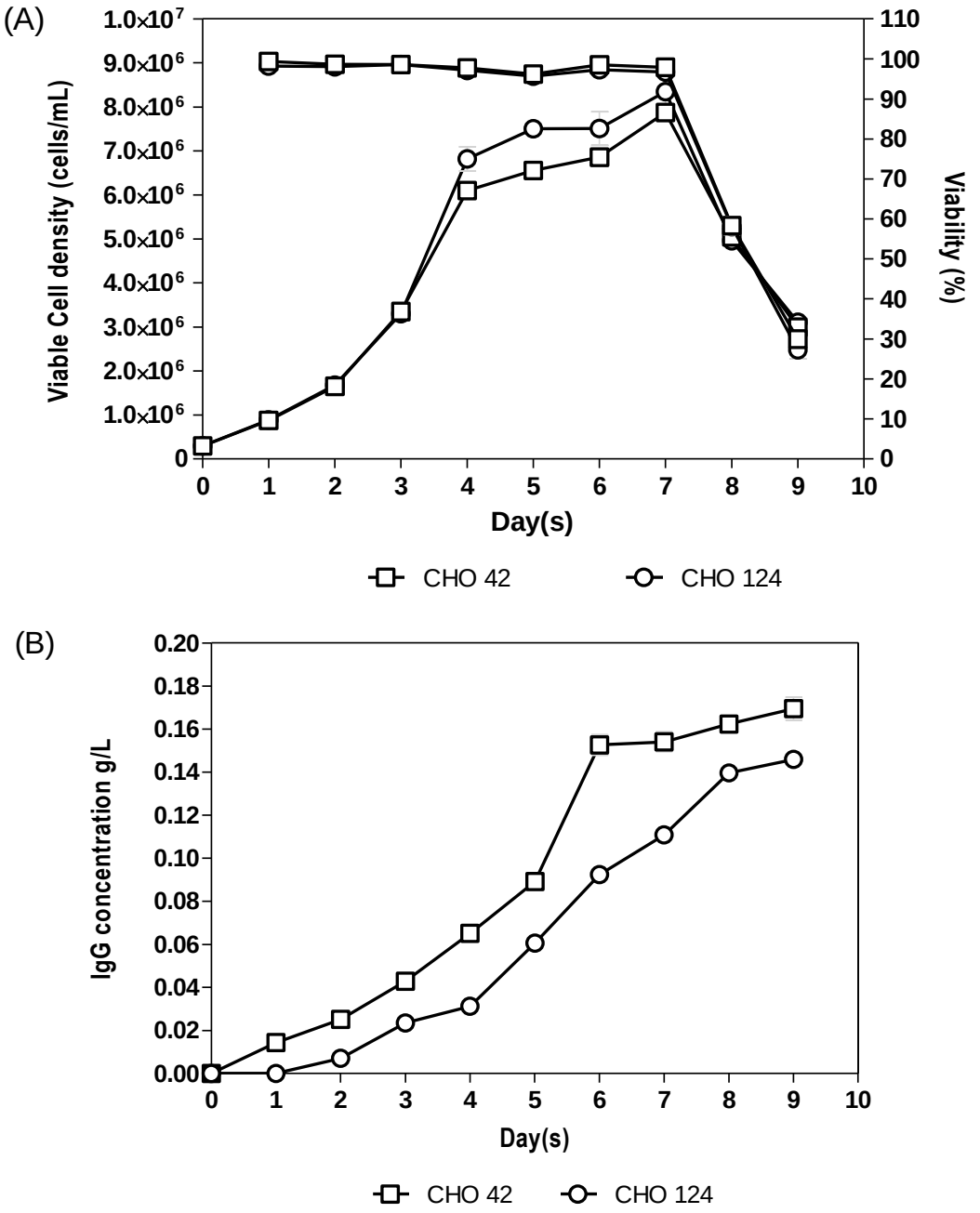


Figure 3.1 Growth characterisation of GS-CHO (CHO42) and GS-CHO (CHO124) in shake flask working volume of 30 mL cultured in CDCHO media + MSX, 5% CO_2 and 37^{o}C. A) The viable cell density and viability profile. B) MAb concentration. The data presented are the average values of triplicate samples analysed in triplicate + S.D.

42. The doubling time for GS-CHO 124 is slightly shorter compare to GS-CHO 42 which is 21.00 hours versus 21.66 hours.

The initial concentration of glucose in the cultures (day 0) was 5.49 g/L and 5.53 g/L for both GS-CHO 42 and GS-CHO 124 culture (Fig 3.2 A). These were marginally lower compared to the amount of glucose that is in the fresh medium of CD-CHO medium which is 6.60 g/L. The concentration of glucose of both GS-CHO cell lines culture decreased as time progressed. GS-CHO 42 cell line consumed glucose faster than GS-CHO 124 where the concentration of glucose decreased quicker on day 5 of culture. At the end of the culture there is still some glucose left in both cultures of the two types of cell lines which are 1.28 g/L in GS-CHO 124 and 0.52 g/L in the culture of GS-CHO 42. As observed in Fig 3.2(B), the amount of lactate which is the main by-product of the culture increased throughout the culture progress. A starting lactate concentration of 0.16 g/L in the culture of GS-CHO 42 on day 0 increased up to 2.06 g/L at the end of the culture on day 9.The, cultures of GS-CHO 124 have a higher lactate concentration on day 9 which is 2.48 g/L. The lactate concentrations reported in this study were comparable to the those noted in the research of Reinhart *et al.* in 2013 where they mentioned a peak of 2.2 g/L concentration of lactate from CHO cells culture in CD-CHO media. Excess lactate in a culture may cause detrimental effects on the cells. It has been shown that excess amounts of lactate accumulation (< 4g/L) in cell culture may affect the growth profile and productivity of cells (Konakovsky *et al.*, 2016; Ozturk *et al.*, 1992). Much research by bioprocess technologists has attempted to reduce lactate accumulation and also to stimulate the consumption of

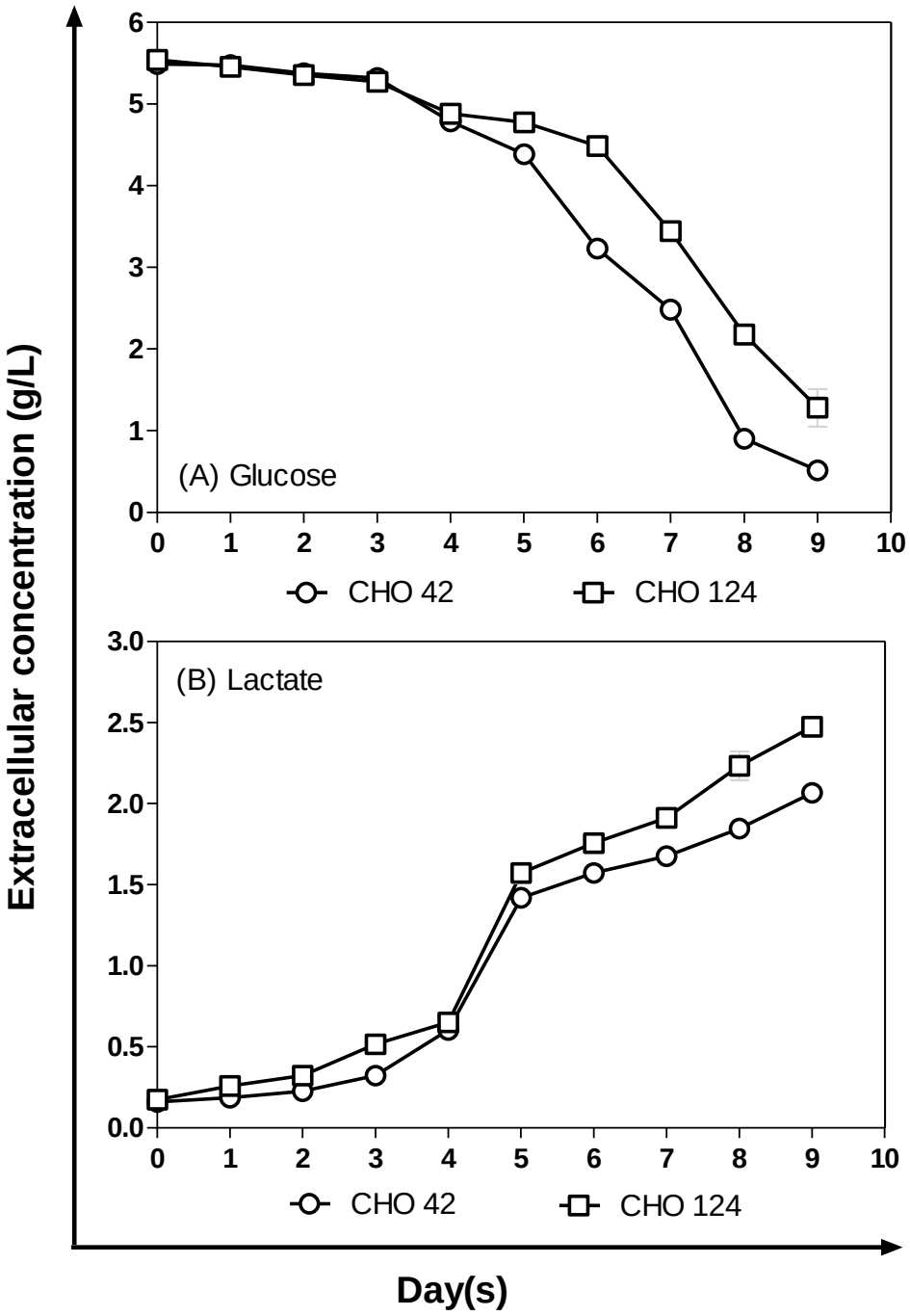


Figure 3.2 Glucose and lactate concentration profiles of main substrate quantified in the culture of GS-CHO (CHO42) and GS-CHO (CHO124) in shake flask working volume of 30 mL cultured in CDCHO media + MSX , 5% CO_2 and 37^{o}C. The data presented are the average values of triplicate samples analysed in triplicate + S.D.

lactate in a cell culture. Li *et al.*, (2012) expressed the opinion that feeding a culture with lactate may allow the by-product to represent an alternative carbon source in certain stages of the culture. They reported that in the rapid growth stage of a cell culture, normally alanine will be actively accumulated. If lactate is depleted in a culture, this will affect the TCA cycle where lactate was usually converted into pyruvate as the alternative carbon source. In order to maintain the flow of TCA cycle, alanine will be converted back to pyruvate as illustrated in Figure 3.3. The conversion of alanine to pyruvate involves two main reactions either via alanine transaminase or alanine dehydrogenase. Both conversions using either enzymes will result in net production of ammonia. Therefore, their study concluded that by feeding lactate in a cell culture may reduce ammonium levels.

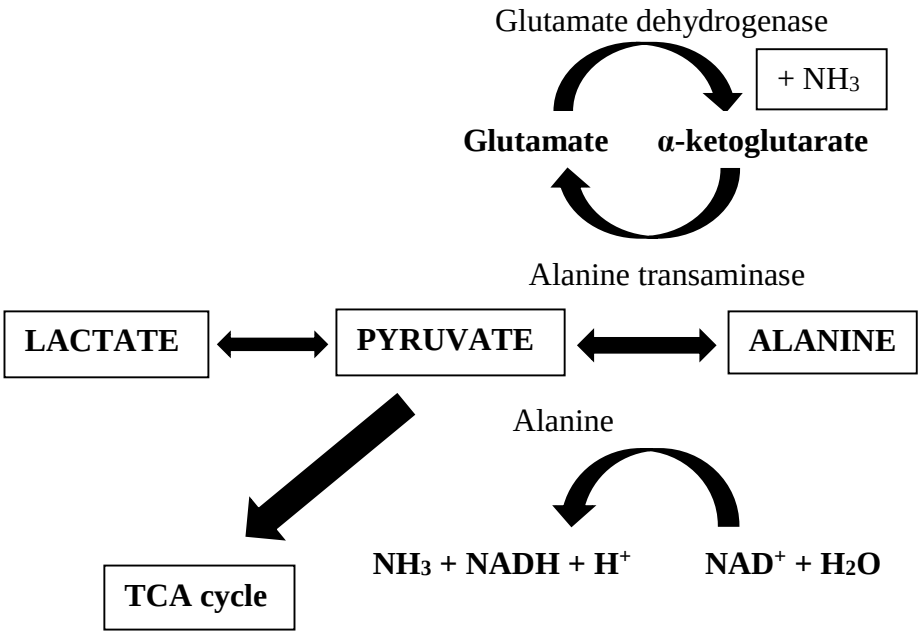


Figure 3.3 Schematics of biochemical reactions in relation with pyruvate, lactate and alanine metabolism. Adapted from Li *et al*., (2012).

Ammonia is another by-product which is formed from glutamine metabolism that may be toxic to the culture. An increased amount of ammonia in a culture (< 4mM) may inhibit the cell growth rate and negatively affect the glycosylation of recombinant proteins (Aghamohseni *et al.*, 2014; Ozturk *et al.*, 1992). Similar to lactate, several researchers have been doing studies on how to reduce or eliminate ammonia in cell cultures. Hong *et al.*, (2010) substituted glutamine with glutamate in the culture of CHO cells. They found that although glutamate may lower the cell growth, it reduced the build-up of ammonia and improved the production of recombinant IgG. Henry and Durocher (2011) examined the HEK-293 cell line containing the yeast pyruvate carboxylase 2 (PYC2) gene in order to enhance the glycoprotein production. It was apparent that lactate and ammonia production were reduced in this cell line and there were also increases in maximum cell density, viable cell concentration and production yield. Then again, Ha and Lee (2014) in an effort to reduce the concentration of ammonia in the culture medium, tried to substitute glutamine with tricarboxylic acid (TCA) cycle intermediates, (alpha-ketoglutarate (α-KG), citric acid and succinic acid) along with glutamic acid for a comparison. The substitution using α-KG showed the best result where final concentration of ammonia did not exceed 3 mM and also led to increased specific productivity and maximum product concentration. Although GS-CHO cell were used in the present study where glutamine is not needed in the culture which will also eliminate ammonia accumulation in the culture; small amounts of ammonia were observed (Fig 3.4 (B)) but only up to 0.09 g/L which will not affect the growth rate of cells and the product yield. As regards the source of this ammonia in such cultures, Carinhas *et al.*, (2013) analysed the metabolic network operation using

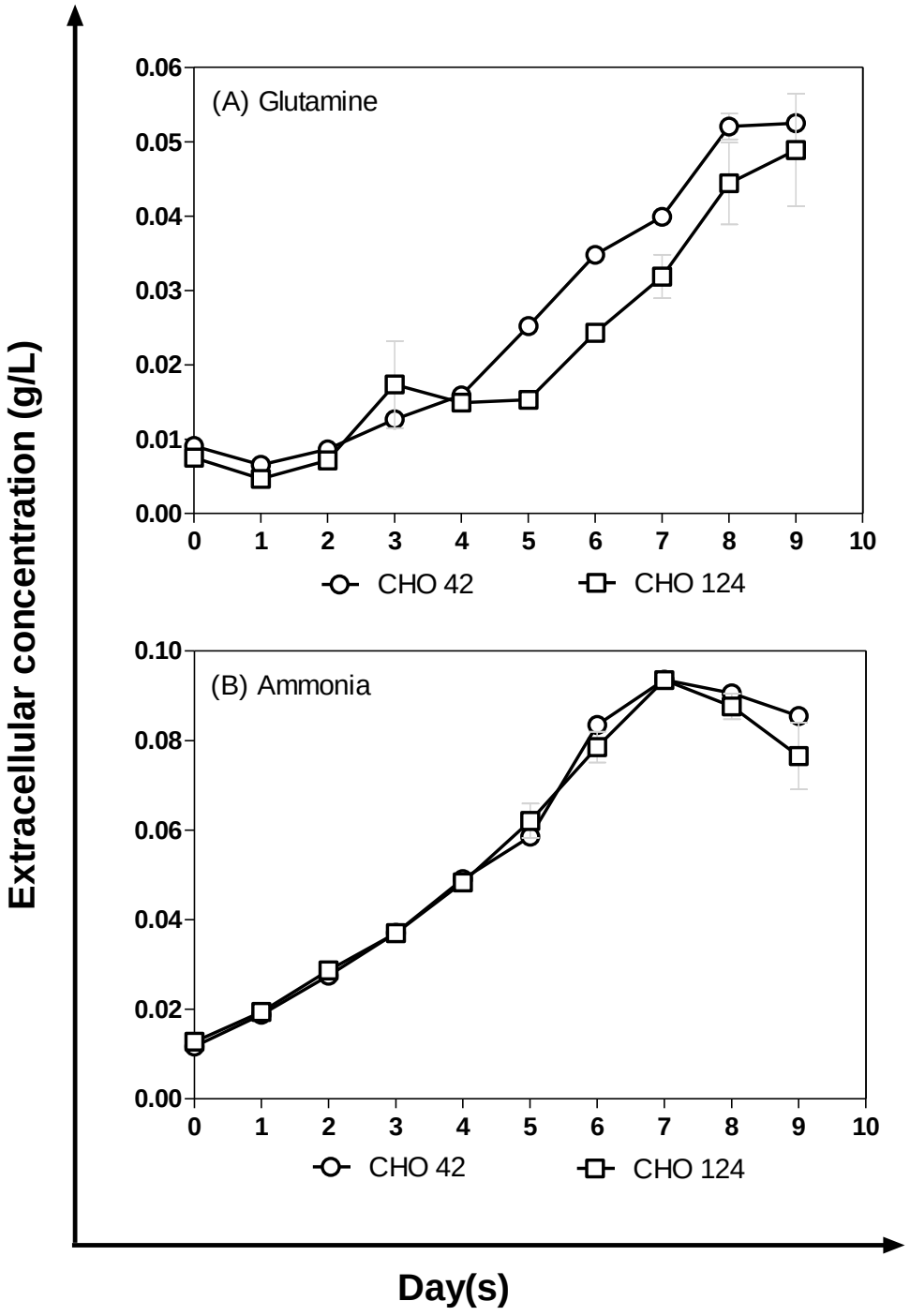


Figure 3.4 Glutamine and ammonia concentration profiles of main substrate quantified in the culture of GS-CHO (CHO42) and GS-CHO (CHO124) in shake flask working volume of 30 mL cultured in CDCHO media + MSX , 5% CO_2 and 37^{o}C. The data presented are the average values of triplicate samples analysed in triplicate + S.D.

^{1}H-nuclear magnetic resonance (^{1}H-NMR) to analyse the supernatant of GS-CHO cell lines. The exometabolomic data revealed that with regard to nitrogen metabolism, uptake of asparagine was intracellularly deaminated to aspartate generating ammonia. As GS cells were used in the present study, addition of exogenous glutamine to the culture medium is not required, because it will be intracellularly synthesized through the GS activity to the amount roughly needed for the growth and productivity necessities. In this study, glutamine levels increased throughout the culture period and reached the highest concentration in the GS-CHO 42 culture of 0.05 g/L (Fig 3.4 (A)). Formerly Carinhas *et al.,* (2013) observed an increased amount of glutamine in their study involving GS-CHO cells where it reached about 1.169 g/L. Similarly Scott (2011) also stated that an excess amount of glutamine was accumulated in the culture medium, which reached 0.55 g/L. However, the amount of glutamine did not affect the growth of cells and the antibody productivity. Other than those mentioned metabolites, glutamate decreased in both cell lines culture medium. This is because glutamate is being utilised to synthesise glutamine. Due to the fact that there was no significant difference in terms of growth rate of both cell lines, GS-CHO 42 cell line was chosen to continue with this research study.

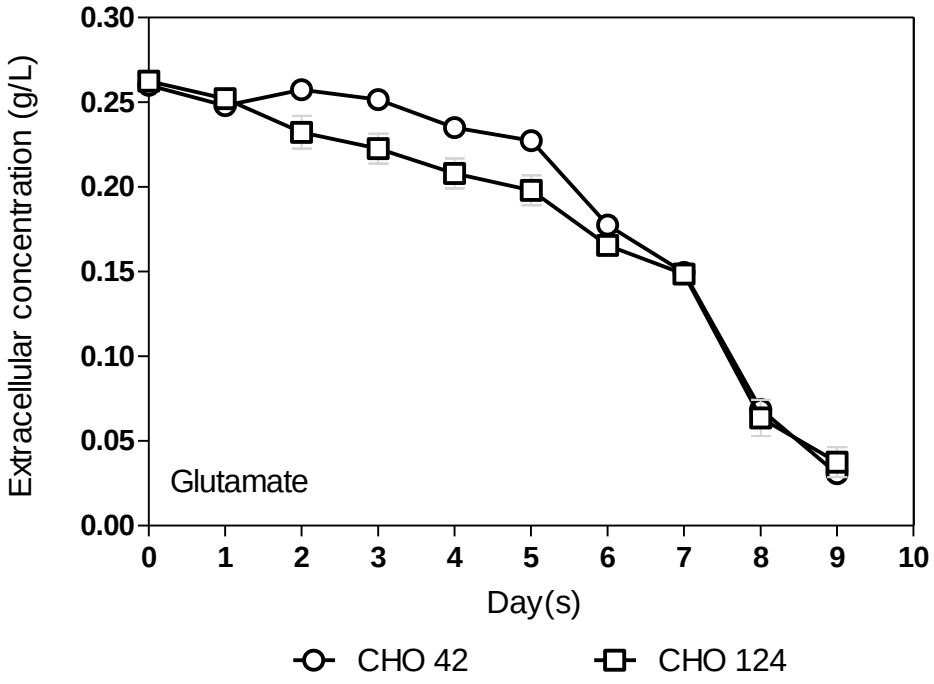


Figure 3.5 Glutamate concentration profiles of main substrate quantified in the culture of GS-CHO (CHO42) and GS-CHO (CHO124) in shake flask working volume of 30 mL cultured in CDCHO media + MSX , 5% CO_2 and 37ºC. The data presented are the average values of triplicate samples analysed in triplicate + S.D.

3.3.2 Characterisation of different passage numbers and different culture media of GS-CHO 42.

In the second part of this chapter, the characterisation of different passage numbers; 4 and 17 of a same cell line (GS-CHO 42), and on different commercially available media (CD-CHO and CD-OPTICHO) during the culture process were being observed. The impact those two parameters, (passage numbers and culture medium) on cell growth and antibody concentrations, as well as the process duration, was evaluated (Fig. 3.6 (A), (B)). A summary of the data generated from the batch cultures is given in Table 3.2.

Table 3.2 Process relevant data from batch culture in shake flask for different passage numbers of GS-CHO 42 (4 and 17) and different culture media.

Passage number	**4**		**17**	
Media	CD-CHO	CD-OPTICHO	CD-CHO	CD-OPTICHO
Peak cell conc. (10^6 cell/mL)	10.74	9.69	8.82	10.20
Antibody conc. (g/L)	0.24	0.11	0.21	0.09
Specific growth rate, μ (h^{-1})	0.040	0.047	0.039	0.044
Doubling time	17.33	14.75	17.77	15.75

Figure 3.6 shows the growth profile for GS-CHO 42 with passage numbers 4 and 17 cultured in two different commercially available media CD-CHO and CD-OPTICHO. The lower passage number cultured in CD-CHO media had the highest cell density of 10.74 x 10^6 cells/mL. However, the peak of cell growth was only observed in day 7, while GS-CHO 42 passage 4 and 17 cultured in CD-OPTICHO media showed an early peak of cells on days 5 and 6. Cells cultured in CD-CHO media showed an extended log phase. Meanwhile cells cultured in CD-OPTICHO media enter the decline phase earlier than cells cultured in CD-CHO media which on day 6 for passage 4 and day 7 for passage 17. Not many research studies on the importance of passage number have been published, although dialogue with the industrial partners suggests this is a real issue for antibody producers. However, (Beckmann *et al.*, 2012; Heinrich *et al.*, 2011) stated that using lower passage number gives several advantages to the cells growth performance and antibody production.

Figure 3.7 shows the IgG production for GS-CHO 42 passage 4 and 17 cultured in CD-CHO and CD-OPTICHO medium. A higher productivity can be seen from GS-CHO 42 passage 4 cultured in CD-CHO media which reached 0.24 g/L and followed by production of 0.20 g/L from GS-CHO 42 passage 17 also cultured in CD-CHO media. Production of IgG from cells cultured in CD-OPTICHO media were both 0.11 g/L and 0.09 g/L. The results for maximum cell density cultured in CD-OPTICHO media were comparable to the study by Carrillo-Cocom *et al.*, (2015) which 7.68 x 10^6 cells/mL; while the mAb production was higher, 0.9 g/L. Study by Sellick *et al.*, (2011) also showed similar number in maximum cell density which was 8.2 x 10^6 cells/mL;

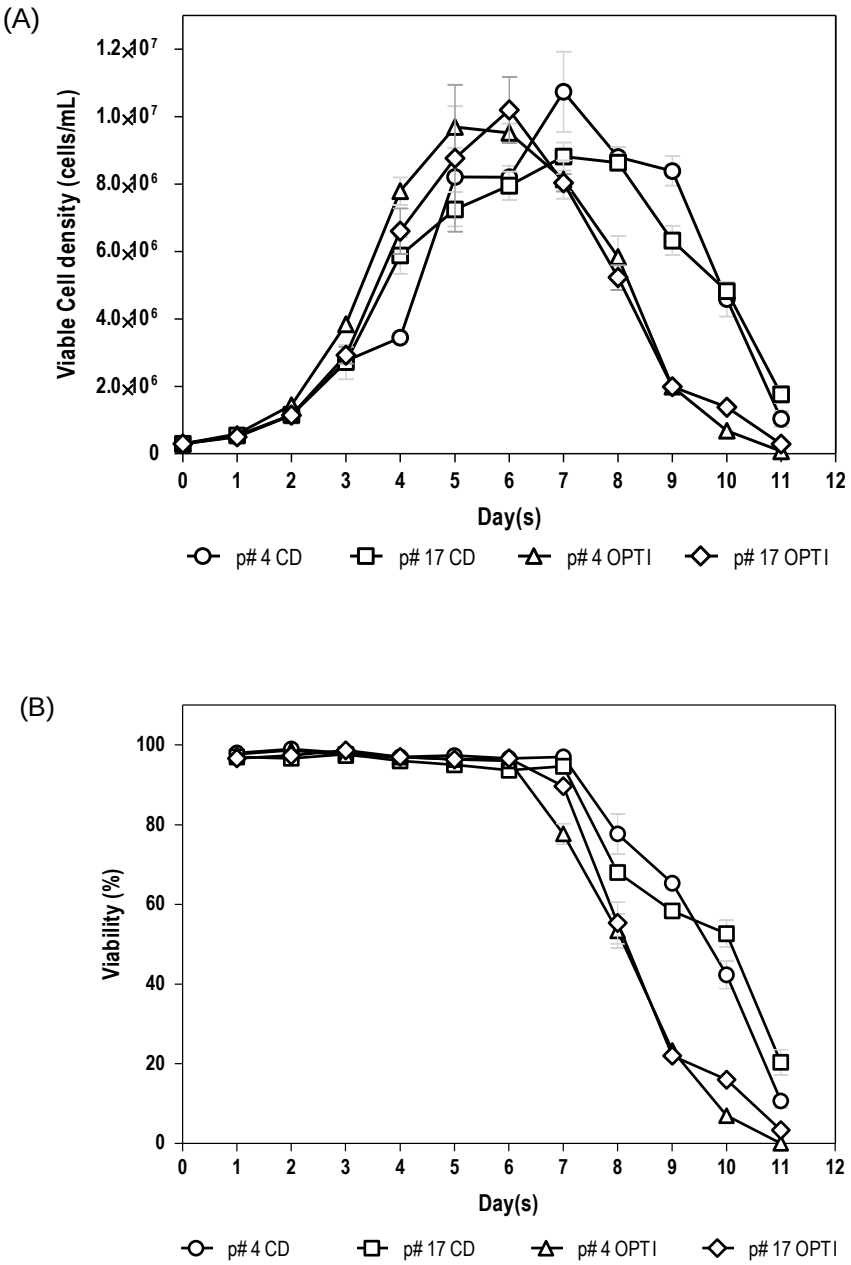


Figure 3.6: Growth of the GS-CHO (CHO42) passage number 4 and passage number 17 shake flask working volume of 30 mL cultured in CDCHO media and CDOPTI media + MSX , 5% CO_2 and 37ºC. A) The viable cell density. B) The viability. The data presented are the average values of triplicate samples analysed in triplicate + S.D.

also with a higher antibody production of 0.4 g/L. On the contrary, Reinhart *et al.*, (2015) reported that cells cultured in CD-OPTICHO media have a higher maximum cell density (4 x 10^6 cells/mL) compared to cells cultured in CD-CHO media (2.5 x 10^6 cells/mL). Then again, in that study the antibody production was also influenced by the cell culture and cell density where, an amount of 0.5 g/L antibody was produced by cells cultured in CD-OPTICHO media while only 0.33 g/L antibody produced by cells cultured in CD-CHO media.

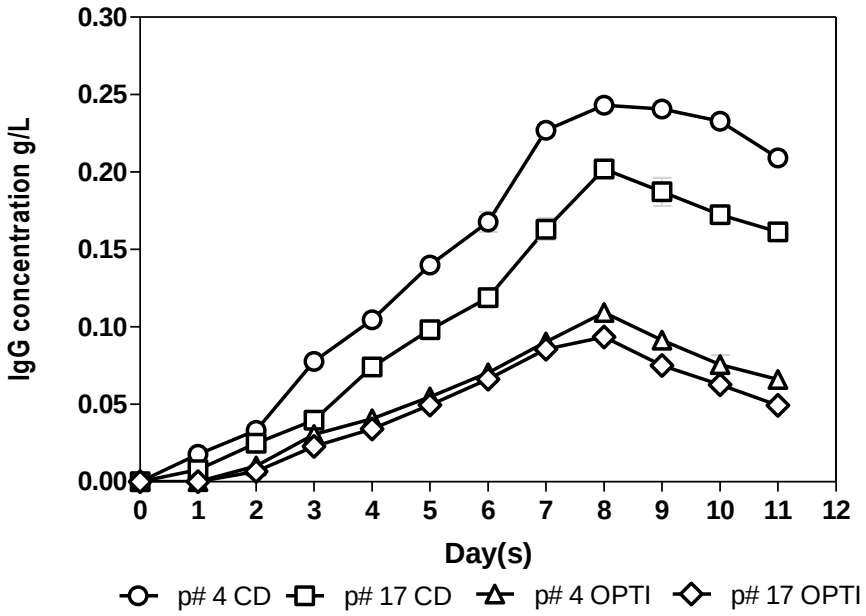


Figure 3.7 Mab profile of the GS-CHO (CHO42) passage number 4 and passage number 17 shake flask working volume of 30 mL cultured in CDCHO media and CDOPTI media + MSX , 5% CO_2 and 37^{o}C. The data presented are the average values of triplicate samples analysed in triplicate + S.D.

Table 3.2 shows the specific growth rate and cell doubling time for GS-CHO 42 passage number 4 and 17. The results for specific growth rate are in the range of 0.039–0.047 h^{-1}. However the results are not significantly different tested by one way ANOVA. Then again, doubling time for GS-CHO 42 passage 4 and 17 culture in CD-CHO and CD-OPTICHO medium were also not significantly different as tested in one way ANOVA. Doubling time were in the range of 14.75 - 17.77 h and were estimated based on the data collected from day 0 to day 4. Even though the findings for lower and higher passage number of GS-CHO cell lines were not significant, the results can be used a s a guide for further investigation of the effect of passage number in a larger scale culture condition for example in the bioreactor. The knowledge can also be used by other researcher who might be using the same cell lines, where passage number 17 can still be used in order to achieve an acceptable cell density and antibody production. Usually, serum free media compose of high concentration of glucose and glutamine which are important for cell growth. Lactate has long been known as the waste product from glucose metabolism. Lactate production did not differ considerably among the tested medium. However, in Figure 3.8 (B) production of lactate increased on day 6 in cultures in CD-OPTICHO media. In fact, the production of lactate was higher compared to cells cultured in CD-CHO media where it reached a peak of 2.56 g/L on day 6. This was slightly higher compared to the levels reported by Reinhart *et al.*, (2015) of 1.8 g/L lactate production from cells cultured in CD-OPTICHO media. However, they also noted lactate being subsequently consumed in the culture and this was also seen in the present study where decline in lactate can be seen after day 7 from both cells of passage 4 and 17 cultured in CD-OPTICHO media. As can be seen from Figure 3.8 (A), glucose concentration in culture of cells in CD-OPTICHO media

declined earlier compared to CD-CHO media. Ammonia levels increased throughout the culture period for both media at first, but dropped in CD-OPTICHO media after day 7 (Figure 3.9 (B)). Glutamine increased in CD-CHO media up to 0.06 g/L using passage 4 cells and 0.04g/L passage 17 cells while maintained at concentration of below 0.02 g/L in CD-OPTICHO (Figure 3.9 (A)). While in Figure 3.10 it can be seen that glutamate decreased with culture time in CD-CHO media while in cultures of CD-OPTICHO medium it increased at first and decreased after day 5.

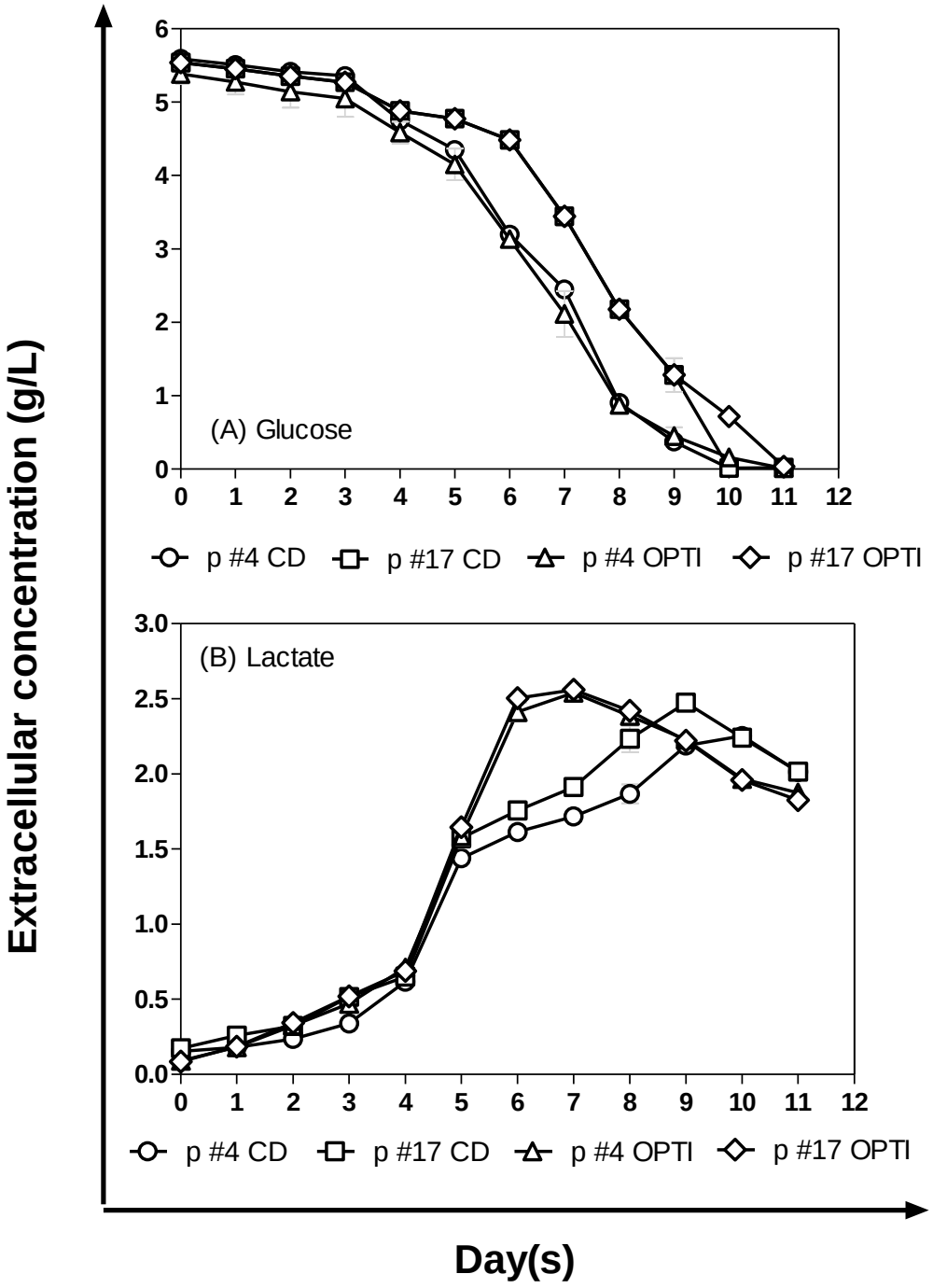


Figure 3.8 Glucose and lactate concentration profiles of main metabolites quantified in GS-CHO (CHO42) passage number 4 and 17 in shake flask working volume of 30 mL cultured in CDCHO and CD-OPTICHO medium + MSX , 5% CO_2 and 37°C. The data presented are the average values of triplicate samples analysed in triplicate + S.D.

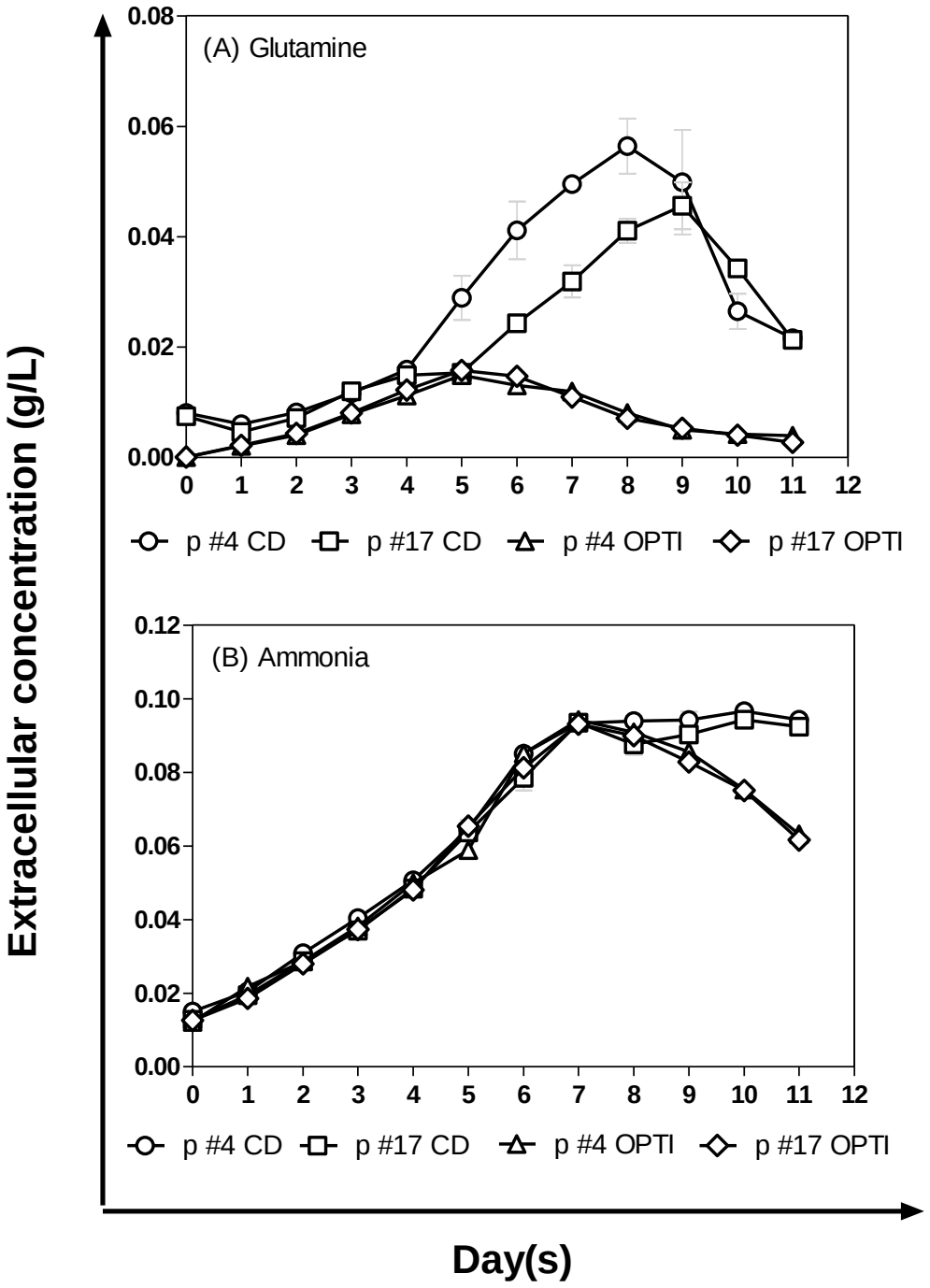


Figure 3.9 Glutamine and ammonia concentration profiles of main metabolites quantified in GS-CHO (CHO42) passage number 4 and 17 in shake flask working volume of 30 mL cultured in CDCHO and CD-OPTICHO medium + MSX , 5% CO_2 and 37°C. The data presented are the average values of triplicate samples analysed in triplicate + S.D.

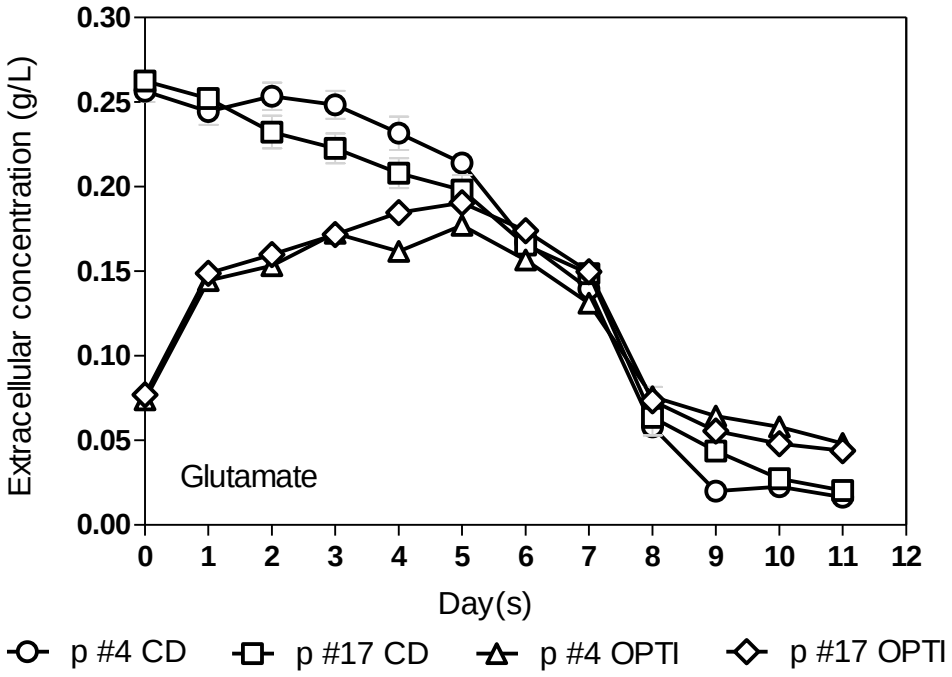


Figure 3.10 Glutamate concentration profiles of main metabolites quantified in GS-CHO (CHO42) passage number 4 and 17 in shake flask working volume of 30 mL cultured in CDCHO and CD-OPTICHO medium + MSX , 5% CO_2 and 37ºC. The data presented are the average values of triplicate samples analysed in triplicate + S.D.

3.4 Conclusion

Characterisation of physiology for the industrial GS-CHO cell lines from different clones and passage number cultured in different types of media in a small scale (shake flask) was successfully achieved and is described in this chapter. A series of reproducible fermentations was carried out with IgG4-expressing GS-CHO cell line in a 125 mL shake flask working volume of 30 mL with different types of cells, different passage numbers and different commercially available growth media. In each type of culture, satisfactory cell growth was achieved but the performance in a high producer cell line with lower passage number was better compared to lower producer and higher passage number. Culture using CD-CHO media showed higher cell density and antibody production relative to CD-OPTICHO media.

These shake flask studies show clearly that medium type and passage number had effects in these industrial cell lines. However, such culture systems may have a number of limitations which might limit the direct relevance of such findings to the bioreactor environment, with its improved process control. The following chapter examines bioreactor culture of these cell lines in detail.

Chapter 4

Characterisation of physiology of industrial CHO cell lines in bioreactor batch cultures

4.1 Introduction

As discussed in the previous chapter, shake flask cell culture is a commonly used method for early stage bioprocess development, and can be used to predict cell line performance at higher scale, for example, in small bioreactor systems. Normally, cell lines which were high producers in shake flask cultures will also give similar results when cultured in bioreactor. Nevertheless, differences between the physics of the two systems may provide changes in the rank position of the cultured cell lines. Dorai (2013) tried to optimize the process of GS-CHO cell lines for high productivity by taking two approaches culturing the cell lines under a variety of culture conditions using Design of Experiment software and proteomic analysis of the bioreactor cultures. From the proteomic analysis study, 12 proteins were found to have process related functions that related to cytoskeleton rearrangement, protein synthesis, cell metabolism and cell growth, and increased understanding of these may at least give new insights into the process optimization work.

On the other hand, Sieck *et al*., (2012) developed a scale down model of the hydrodynamic stresses present in large scale production bioreactors to investigate the performance of CHO cells under simulated production bioreactor conditions. Stirring and sparging are necessary at large scale to supply the cells in culture with oxygen and nutrients for growth and production, they are also the source of hydrodynamic stress that could cause cells damage or affect cell performance. Various levels of hydrodynamic stress were generated in 2 L bioreactors mimicking large scale stirred tank bioreactor. Hydrodynamic stresses were commonly quantified using the energy dissipation rate, ε (W/kg), a scalar value that describes the irreversible conversion of

kinetic energy to heat and depends on the position inside the vessel. Agitation rates changing periodically in time were applied to mimic the repeated exposure of cells to different local values of ε present in different locations inside the large scale bioreactor. The local ε values were derived from theoretical analysis and literature values. Hydrodynamic experimental conditions in the 2 L scale-down studies were 0.001, 0.01, 0.06, 0.4, 0.01-0.4 and 1.015 W/kg which simulated large scale bioreactor compartments from low turbulence zone, bulk zone, standard 2 L agitation rate, high turbulence zone, scale down model and tip speed of production scale. Their findings showed that viable cell density of CHO cells is very comparable for ε ranging from 0.01 to 1.015 W/kg. From the results also they stated that the cell line that was used was highly robust against agitation shear stress, even in comparison with other industrial cell lines. However, specific productivity of monoclonal antibody shows a slight decrease at high hydrodynamic stress (0.4 W/kg) where a reduction 25% was observed. Conversely no changes in metabolite consumption or by product formation were observed.

In the same way, Nienow *et al*., (2013) investigated the physical characteristics of a microscale bioreactor system with a working volume of 15 mL using an industrial CHO cell line. Results showed a similar cell growth and productivity to that achieved in a 5 L stirred tank bioreactor despite being significantly different from those observed in cells cultured in shake flask. It was proposed that the similarity of the results between micro and 5 L scale systems were due to the similar control capabilities and the equivalence of the stress parameters across the scales when compared with shake flask.

As mentioned in Chapter 3, shake flask studies were carried out in order to look at the characterisation of industrial GS-CHO cell lines in small scale culture. However, in order to gain more insight into the capabilities of the industrial CHO cell lines in producing the monoclonal antibody, it is very important that the cell lines can grow in a larger scale and also in a more controllable environment in the bioreactor. Therefore, this chapter will focus on the characterisation of physiology for the industrial CHO cell lines at different growth phases in bioreactor batch cultures.

4.2 Materials and Methods

GS-CHO 42 passage number 7 were cultured in three different commercially available CHO cell media; CD-CHO, CD-OPTICHO and Dynamis which have been kindly provided by ThermoFisher Scientific. Passage 7 is the lowest passage number of a cell line that can be used to inoculate into the bioreactor as illustrated in Figure 2.5. Batch cultivations were performed in 5 L bench top bioreactors with a 3 L working volume and the parameters for bioreactor setup were as in section 2.10. The cell numbers were determined by the trypan blue exclusion methods using haemocytometer (Section 2.4) and the antibody concentrations were determined using ELISA, routinely throughout the culture duration until the cells reached death phase (Section 2.10.2).

4.3 Results and Discussion

In batch experiments of the bioreactor, the impact of the different culture media on cell growth and antibody concentrations, as well as the process duration, was evaluated (Fig. 4.1 (A), (B)). A summary of the results from the batch cultures is given in Table 42.1.

Table 4.1 Process relevant data from batch culture in 5 L bioreactor working volume of 3 L for GS-CHO 42 cultured in three commercially available medium.

Cell Type	**CD-CHO**	**CD-OPTICHO**	**Dynamis**
Peak cell conc. (10^6 cell/mL)	5.83	2.48	1.98
IgG conc. (g/L)	0.37	0.15	0.24
Specific growth rate, μ (h^{-1})	0.032	0.025	0.024
Doubling time (h)	21.66	27.73	28.88

The viable cell density and viability profiles are shown in Figure 4.1. The growth pattern differed markedly between different media. GS-CHO cells cultured in CD-CHO media grew fast during exponential phase (0.032 h^{-1}) and peaked after 7 days at a high cell density (5.83 x 10^6 cells/mL). Cells in CD-OPTICHO medium grew slightly slower as cells in CD-CHO media in the exponential phase (0.025 h^{-1}) but entered stationary phase around day 5 at a low density of only 2.48 x10^6 cells/mL. Despite that, the specific growth rate for GS-CHO cultured in the three different medium were not significantly different tested by one way ANOVA where cells in Dynamis medium grew at 0.024 h^{-1} during exponential phase with a final cell density of only 1.98 x 10^6 cells/mL on day 7. The viability dropped below 95% on day 3 in CD-OPTICHO and Dynamis medium, while in CD-CHO cultures, viability remained above 95% until day 7 of culture. There are not many research studies of CHO cells using the CD-OPTI medium in batch mode using a bioreactor, however a study by Gonzalez-Leal *et al*., (2011) and Lopez-Meza *et al*., (2016) showed a higher density of cells were obtained compared to this study which are 4.5 +/- 0.5 x 10^6 cells/mL and 4.00 x 10^6 cells/mL accordingly; compared to the peak of cells in this study. With Dynamis media which was a recently developed medium at the time of this study, there was little or no available literature in peer reviewed journals which would have allowed to a comparative assessment of the growth profile, yet the manufacturer stated that Dymanis medium offers the highest batch performance and yield of recombinant CHO cells. Therefore, the present study can be a foundation to evaluate this new commercially available chemically defined medium for culturing GS-CHO cells in other future studies. Based on the findings of the present study, GS-CHO cells can be grown in other media than CD-CHO (the normal medium used) with acceptable

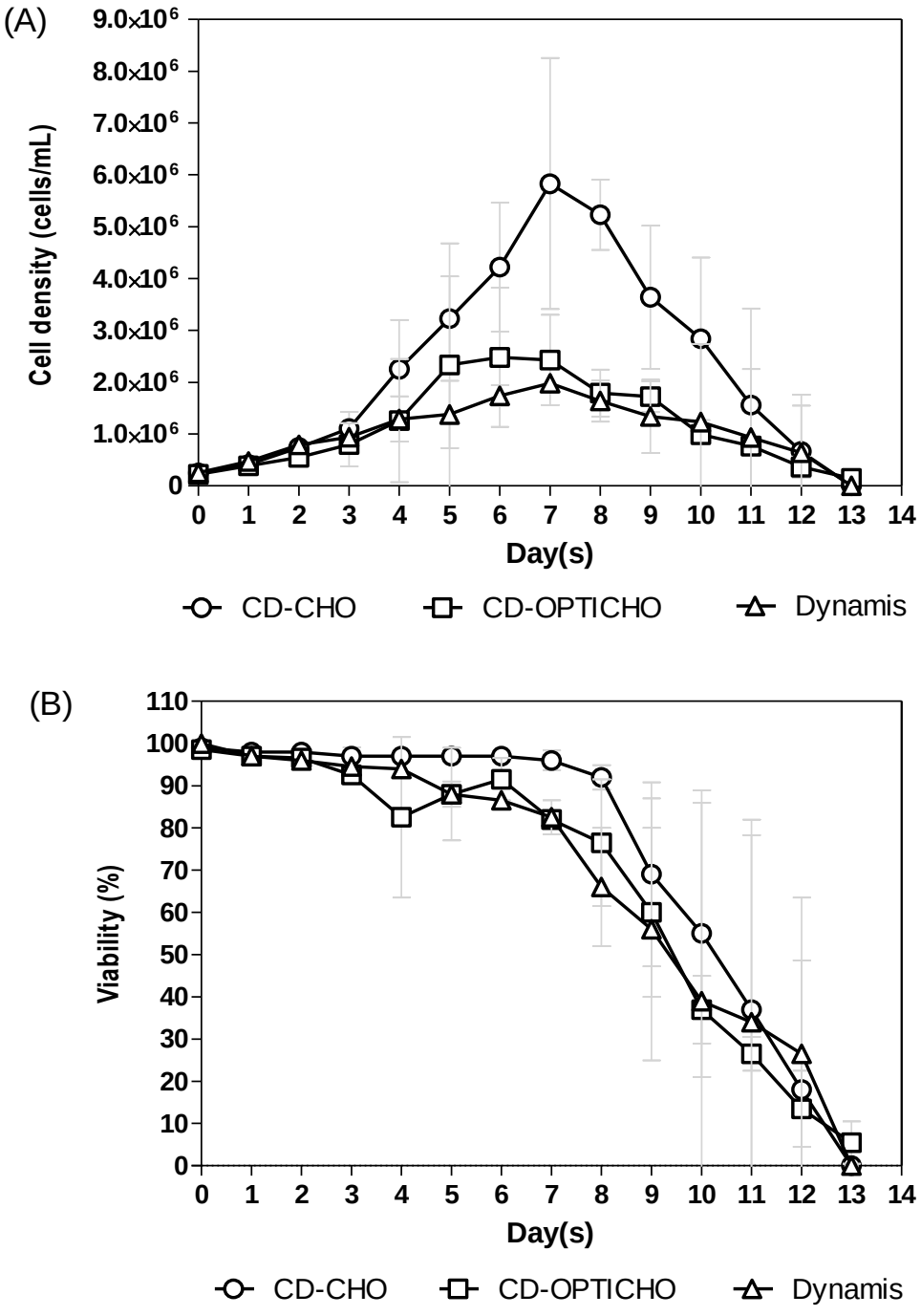


Figure 4.1 Growth characterisation results of GS-CHO (CHO42) passage 7 in bioreactor working volume of 3 L cultured in CD-CHO, CD-OPTICHO and Dynamis medium + MSX, 5% CO_2 and 37ºC. A) The viable cell density profile. B) Viability profile. The data presented are the average values of duplicate samples analysed in duplicate + S.D.

density and viability throughout the culture period when adaptation time was given longer. Cells need to be subculture several time into the new medium (CD-OPTICHO and Dynamis) in order to obtain a better growth performance as claimed by the manufacturer.

The IgG concentration showed a maximum level of 0.37 g/L on day 10 for cells cultured in CD-CHO media. While GS-CHO cultured in CD-OPTICHO and Dynamis medium showed a maximum titre of 0.15 g/L and 0.14 g/L of IgG on day 11 before an increase up to 0.24 g/L of IgG from cells cultured in Dynamis. The IgG concentration in CD-OPTICHO medium in the present study was higher compared to the figure reported in the study by Gonzalez-Leal *et al*., (2011) where a maximum of only 0.018 g/L of mAb was achieved. Similarly, in a study by Lopez-Meza et al., (2016) only 0.015 g/L mAb was produced. Although some of the parameters used in the two research study mentioned above are not the same as in this study, it can be a useful guideline in order to compare the results of their study to this study. This is because, both studies were in a batch mode in bioreactors and both used CD-OPTICHO as their base medium. Table 4.2 shows the differences between three research studies. In the present study the differences in IgG production from cells cultured in the three commercially available culture media are significant as tested by one way ANOVA.

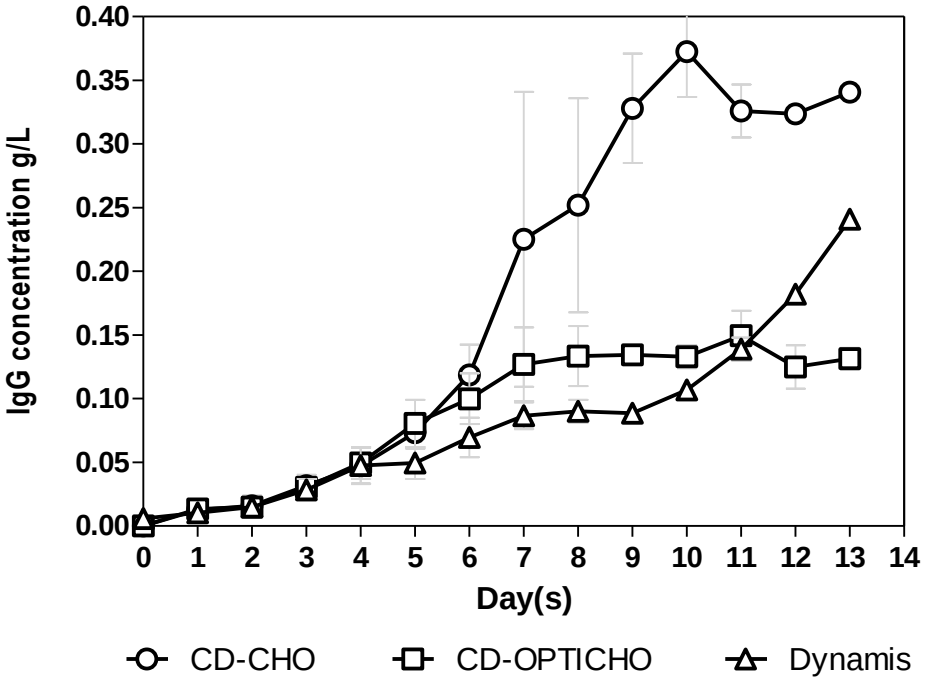


Figure 4.2 MAb concentration results of GS-CHO (CHO42) passage 7 in bioreactor working volume of 3 L cultured in CD-CHO, CD-OPTICHO and Dynamis medium + MSX, 5% CO_2 and 37ºC. The data presented are the average values of duplicate samples analysed in duplicate + S.D.

Table 4.2 Comparison of studies using CD-OPTICHO medium as the base medium for CHO cells.

Studies	**Gonzalez-Leal *et al*., (2011)**	**Lopez-Meza *et al*., (2016)**	**This study**
Cell line used	CHO-DG44 producing monoclonal antibody (Invitrogen, A11000-01; Life Technologies, Carlsbad, CA.	CHO-S clone producing a biosimilar of the monoclonal antibody infliximab and its native counterpart (CHO-S line from Invitrogen, Carlsbad, CA, USA).	GS-CHO producing cB72.3 IgG4 monoclonal antibody provided by Lonza Biologics (Slough, UK).
Culture mode	Batch culture in 2-L bioreactor (EZ Control from Applikon Biotechnology, Schiedam, the Netherlands).	Batch culture in 1.6-L bioreactors (DASGIP, Julich, Germany).	Batch culture in 5 L bioreactor (*Applikon* Biotechnology BV, The Netherlands)
Working volume	1 L	1 L	3 L
What study being conducted	Determining the effect of selected amino acids on monoclonal antibody production in CHO cells using Plackett-Burman statistical design.	Characterize the kinetics of cell growth, substrate consumption, and product formation in naive and monoclonal antibody (mAb) producing recombinant CHO cells.	Characterisation of physiology of industrial CHO cell lines cultured in different types of commercially available culture medium.
Peak cell conc. (10^6 cell/mL)	4.50 +/- 0.5	4.00	2.48
Antibody conc. (g/L)	0.018	0.015	0.15

According to (Nienow et al., 2013) shake flask culture of GS-CHO cells gave higher product titre despite a lower cell density and viability compared to the culture in a bioreactor. This is in contrast to the present study, where the IgG concentrations were higher in bioreactor cultures (Figure 3.7 and Figure 4.2). Table 4.3 shows the comparison between cultures in shake flask and bioreactor cultures with CD-CHO and CD-OPTICHO media. It can be seen that CD-CHO medium produced better results for GS-CHO cells cultured either in shake flask or in bioreactor when compared to CD-OPTICHO. Although cell densities were higher in shake flask cultures than in bioreactors, the mAb concentrations were higher in bioreactor cultures. The amount of cell density and IgG concentration in shake flask culture and bioreactor culture are significantly difference tested by one way ANOVA. Cells were able to grow in CD-OPTICHO medium but may well require a lengthier adaptation time to ensure a high IgG production.

Table 4.3 Process relevant data from batch cultures in shake flask and bioreactor with CD-CHO and CD-OPTICHO media

	Shake flask culture		**Bioreactor culture**	
	CD-CHO	CD-OPTICHO	CD-CHO	CD-OPTICHO
Peak cell conc. (10^6 cells/mL)	10.74	9.69	5.83	2.48
specific growth rate, μ (h^{-1})	0.040	0.047	0.032	0.025
IgG conc. g/L	0.24	0.11	0.37	0.15

.

The extracellular concentration of glucose, lactate, ammonia, glutamine and glutamate were measured during the bioreactor cultures. In relation to the physiology of cell culture and IgG production, monitoring of these five analytes is very important. This is because, glucose is the major carbon and energy source for cultured CHO cells in most cases, however glycolytic metabolism often results in the accumulation of lactate which will adversely affects cell growth and productivity. Balancing the level of glucose in CHO cell culture is very important because if glucose levels fall to limiting levels, the culture may undertake quick degeneration leading to loss of protein. Glucose and lactate levels are therefore routinely measured to determine metabolic activities of the culture. Ammonia on the other hand is a major inhibitory waste product of glutamine metabolism in mammalian cell cultures, and is released by chemical decomposition of glutamine and by metabolic deamination of glutamine to glutamate and by the conversion of glutamate to α-ketoglutarate. Therefore, it also important to monitor the concentration of ammonia and glutamine daily in CHO cell culture. Several studies have been done in order to control the waste level of by-product (lactate and ammonia) (Aghamohseni *et al*., 2014; Chee Furng Wong *et al*., 2005; Duarte *et al*., 2014; Kishishita *et al*., 2015). While for glutamate, study by Hong *et al*., (2010) stated that replacement of glutamine by glutamate improves production and galactosylation of recombinant IgG in Chinese hamster ovary cells. This is because glutamate does not suffer from spontaneous decomposition and, therefore, is inherently less ammoniagenic than glutamine or dipeptides. While according to Zhang *et al*. (2015) glutamate can act as substitute to represent the overall nutrient consumption because glutamate had been shown to be consumed in constant molar ratios compared to other key nutrients. Their study of monitoring of glutamate level

could adjust the complex feed amount during the cell culture process and improve production.

The initial concentration of glucose (day 0) was 6.25 g/L in CD-CHO medium which is the highest concentration among the three media (Fig 4.3 (A)). While the initial concentration of glucose in CD-OPTI and Dynamis were 4.50 g/L and 5.92 g/L respectively. The concentration of glucose in all media declined as the culture progressed. The first culture medium to exhibit exhaustion of glucose was CD-OPTICHO where glucose concentration reached 0 g/L on day 9. This may simply be due to the initial concentration of glucose in the fresh medium (no cells cultured inside it), CD-OPTICHO medium being lower (5.501 g/L) compared to glucose level in CD-CHO and Dynamis which were 6.605 g/L and 6.574 g/L. The early decline of glucose in cultures using CD-OPTICHO medium may have contributed to the increased concentration of lactate in the media. However, as can be seen from Figure 4.3 (B) on day 9 lactate consumption is apparent where the production of lactate have been switch to lactate consumption due to no glucose as the carbon source. This finding is in agreement with research by Lopez-Meza et al., (2016) where they stated that lactate was actively produced during the stage of exponential cell growth for later consumption once the cell density reached its peak. The pattern of lactate concentration in Dynamis culture media was similar to that in the CD-OPTICHO media and also decreased a little towards the end of the culture. The peak concentration of lactate in both CD-OPTICHO and Dynamis medium were 2.52 g/L and 2.88 g/L (Fig 4.3 B). These were slightly higher compared to the lactate concentration from Lopez-Meza *et al*. (2016) study where the maximum lactate concentration did not exceed 1.0 g/L. The lactate levels reported in the present study however are not likely

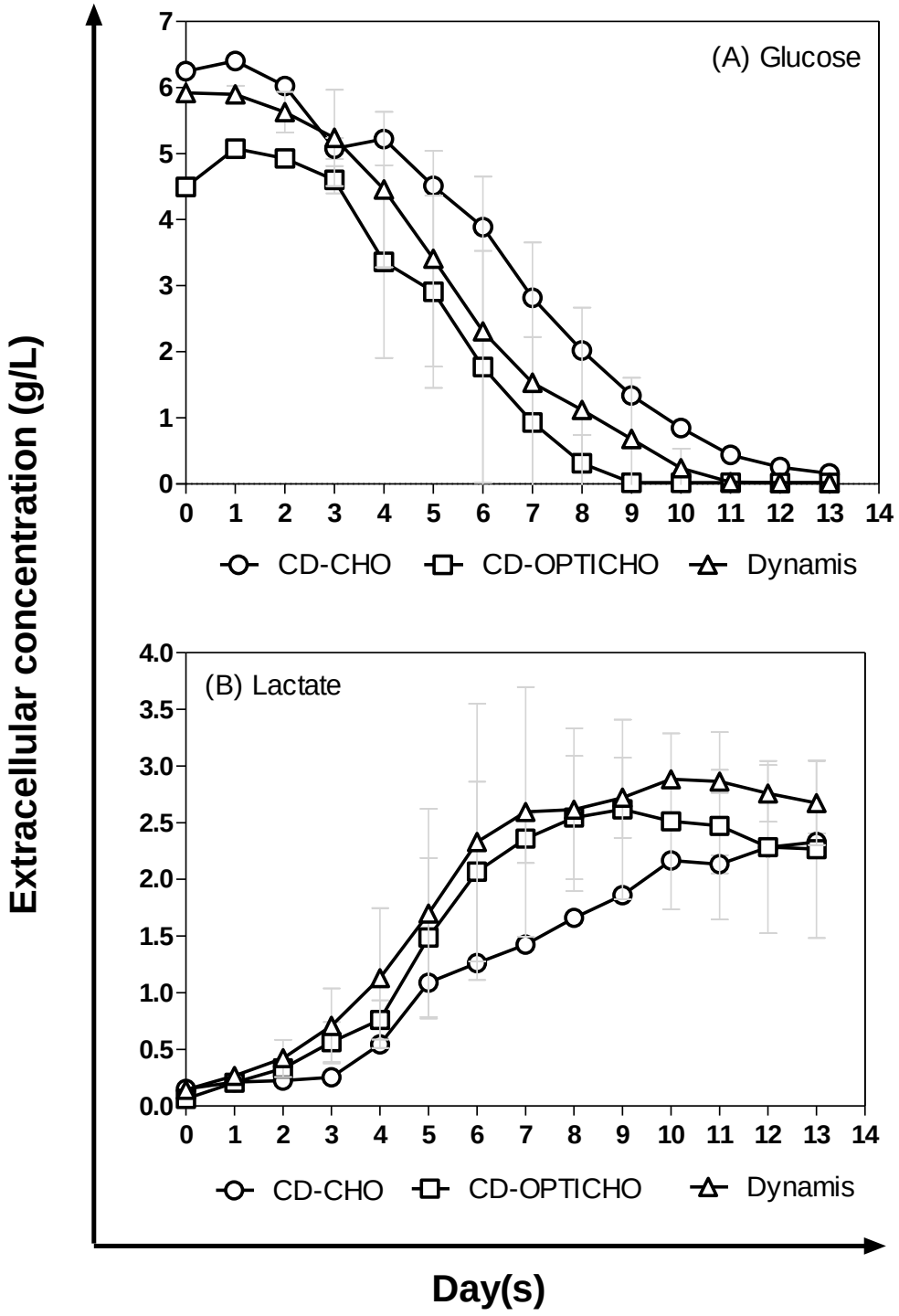


Figure 4.3 Glucose and lactate concentration profiles quantified in the culture of GS-CHO (CHO42) passage 7 in bioreactor working volume of 3 L cultured in CD-CHO, CD-OPTICHO and Dynamis medium + MSX, 5% CO_2 and 37ºC. The data presented are the average values of duplicate samples analysed in duplicate + S.D.

to have affected the culture of GS-CHO cells in term of cell growth and product concentration. In contrast the situation in the above media, lactate concentration in CD-CHO culture medium increased towards the end of the culture up to 2.33 g/L. Cells cultured in CD-CHO medium may not use lactate as their carbon source because some glucose is still available (0.16 g/L) at the end of the culture (day 13). A study by Martinez *et al*., (2013) using CHO cells cultured in CD-CHO media showed a switch from lactate production to lactate consumption. Lactate accumulation in their study reach up to 50 mM which is about 4 g/L before it was switch to consumption by the cells as the carbon source. As mentioned before, Konakovsky *et al*., (2016) and Ozturk *et al*., (1992) reported that lactate of more than 4 g/L will effect cell growth, in the case of my findings, where lactate levels was only at 2.33 g/L, will not be affecting the cell growth performance and antibody production. Ammonia levels increased continually throughout the batch cultures, reaching concentrations between 0.12 and 0.14 g/L (Fig 4.5 (B)). However the concentration of ammonium in this culture study did not affect the cell growth performance and IgG productivity. As can be observed in Fig 4.5 (A), glutamine concentration in the culture of GS-CHO cells in the three different media increased at the beginning and dropped at the middle of culture for CD-OPTICHO (day 5), while at the end of culture for Dynamis (day 8) and CD-CHO media (day 10). The peak concentrations for glutamine were between 0.015 and 0.044 g/L.

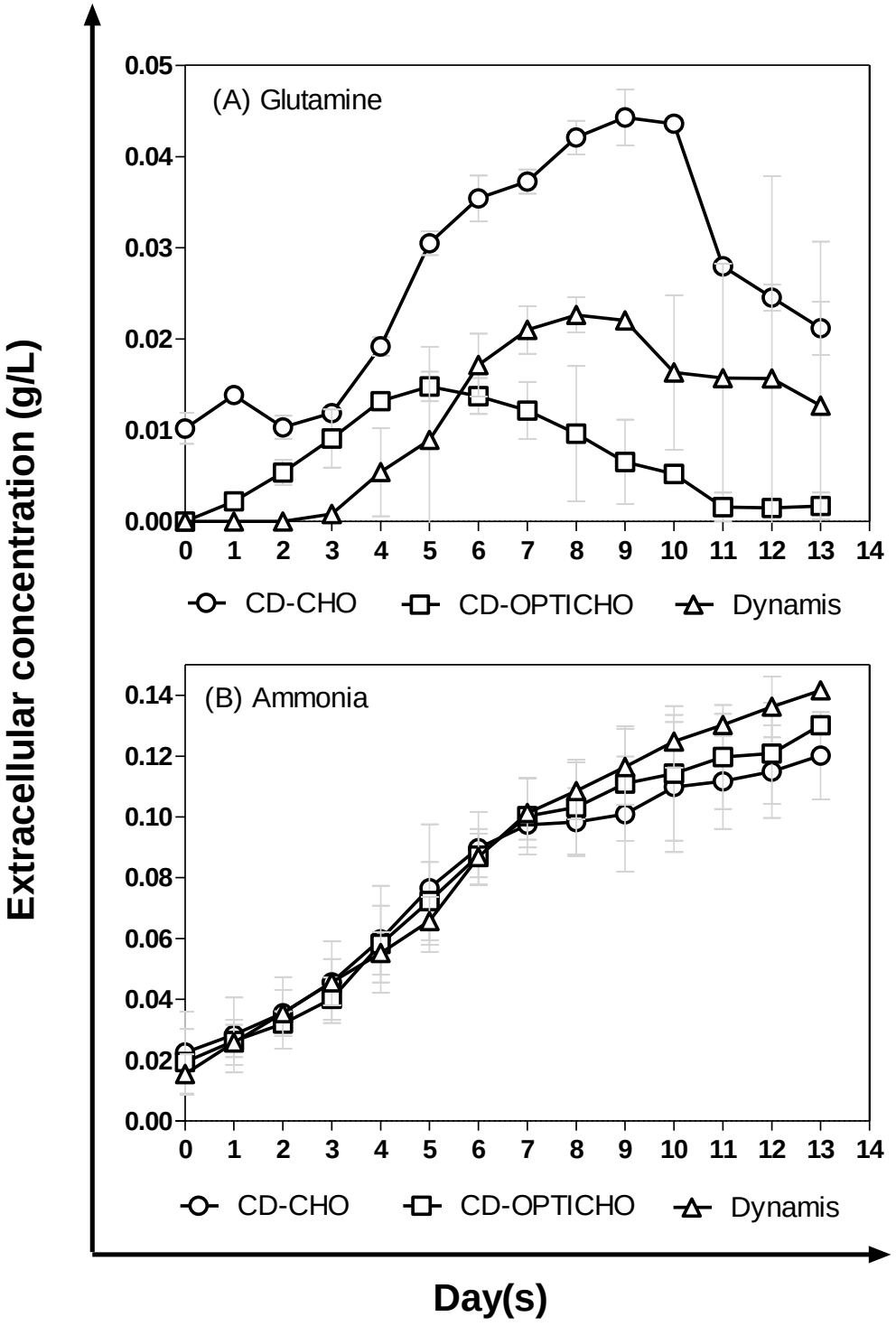


Figure 4.4 Glutamine and ammonia concentration profiles quantified in the culture of GS-CHO (CHO42) passage 7 in bioreactor working volume of 3 L cultured in CD-CHO, CD-OPTICHO and Dynamis medium + MSX, 5% CO_2 and 37ºC. The data presented are the average values of duplicate samples analysed in duplicate + S.D.

Finally, as in Figure 4.5, glutamate concentration went up throughout the culture period in Dynamis medium up to 0.688 g/L (Fig 4.4 E), while it was depleted in both CD-CHO and CD-OPTICHO media. Glutamate is also one of the amino acids that play an important role in a culture media.According to Altamirano *et al*., (2000) by replacing glucose and glutamine (the most commonly employed carbon and nitrogen sources) may lead to a more efficient use of these compound and also decreasing the accumulation of lactate and ammonium in the medium. Their study chooses compounds that are slowly metabolized includes galactose and glutamate that gave the best results among other tested compounds. They mentioned that the fact that glutamate has only one amino group to its structure and the fact that glutamate is transported into the cell at a lower velocity should provide a more efficient use of this compound with lower generation of ammonium. Glutamate also does not suffer spontaneous decomposition as glutamine where ammonium ions will not be generated as a result. In the same way, Hong *et al*., (2010) stated that glutamate can be used as a substitute for glutamine in CHO culture medium. Other than reducing ammonia formation, it can also enhanced the production of recombinant IgG (rIgG). The use of glutamate also increased complement-dependent cytotoxicity activity in galactosylation, one of the effector functions of rIgG.

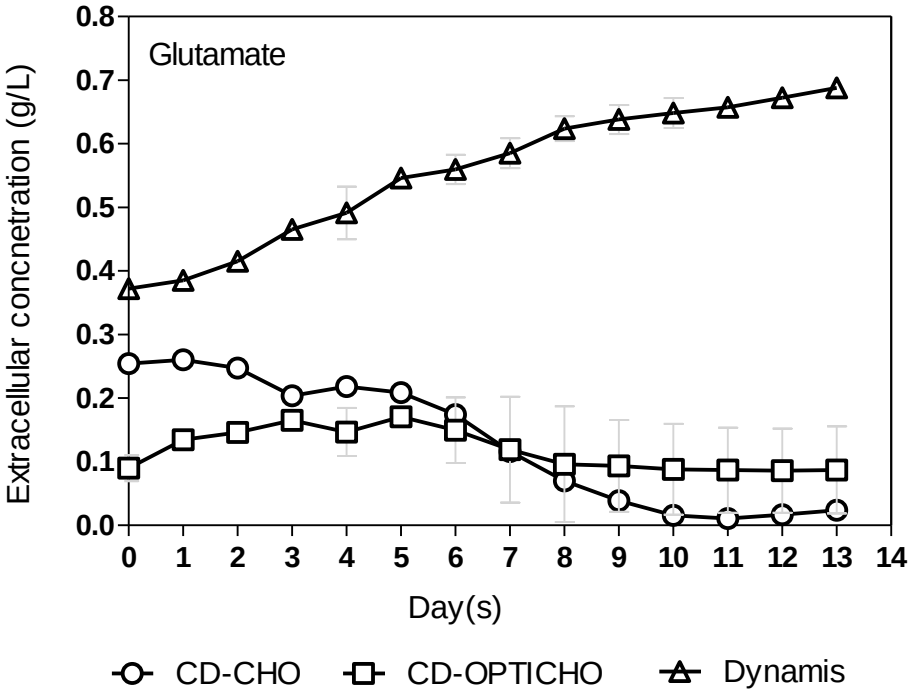


Figure 4.5 Glutamine and ammonia concentration profiles quantified in the culture of GS-CHO (CHO42) passage 7 in bioreactor working volume of 3 L cultured in CD-CHO, CD-OPTICHO and Dynamis medium + MSX, 5% CO_2 and 37^{o}C. The data presented are the average values of duplicate samples analysed in duplicate + S.D.

4.4 Conclusion

A physiological characterisation of the industrial CHO cell line at in bioreactor batch cultures was successfully carried out and is presented in this chapter. Current state-of-the-art of bioreactor systems were used to study and compare the difference of growth between an industrially relevant cell lines. GS-CHO cells grown in three different commercially available chemically defined media. The findings also were compared with the growth in shake flask culture from previous chapter. Results from such analysis can pave the way for the evaluation of metabolites identification associated with cell death or product decrease which will be described in the subsequent chapters.

Chapter 5

Characterisation of the physiology of industrial CHO cell lines in bioreactor fed batch cultures

5.1 Introduction

Among all the culture conditions of cells, fed batch culture has gained popularity since it can deliver higher product titre and extend the productive phase up to three weeks. Nutrient exhaustion in typical batch cultures may negatively affect cell growth and the ability of cells to produce high product titres. Therefore, concentrated feeds are added to the culture to overcome the problem of nutrient limitation. This is very important because any improvement in titre or productivity during cell culture may reduce the cost of drug manufacturing. Many researchers have been studying culturing CHO cells in fed batch mode with many kind of improvements. An increase on cell productivity has been seen from the study by Koumpouras and Kontoravdi (2012) where they present the results of a dynamic optimization approach to improve an animal cell bioprocess and found that the final antibody titre could be improved by up to 70% in a fed batch culture when compared to batch culture. Additionally, Kim *et al*., (2013) mentioned in their study of fed-batch CHO cell culture that an increase to 250 mg/L of tissue-plasminogen activator (t-PA) was achieved compared to only 33 mg/L under batch culture conditions. On the other hand, Toussaint *et al*., (2016) researched into the metabolic engineering of CHO cells, where they used a recombinant yeast pyruvate carboxylase (PYC2) expression in a CHO cells to alter lactate metabolism in a fed-batch culture. This is because fed batch culture often being associated with longer cultivation time, therefore producing high lactate that may cause detrimental effect to CHO cells. The results showed that not only the metabolism of lactate was shifted towards consumption, but also the PYC2 cells are well suitable for fed batch process where it resulted in further significant increases in terms of maximum cell density and

final product titre. From the above mentioned studies, it can be seen that medium and feed supplementation are steadily evolving and both can have intense influence on protein production.

In the present study, we were supplied with two different types of feed supplement sponsored by Thermo Fisher Scientific, namely CHO CD Efficient Feed A (EFA) and CHO CD Efficient Feed B (EFB) feeding supplement. According to the manufacturer, both EFA and EFB were formulated to provide up to 2.5 fold improvement in titres used in fed batch compared to batch culture. The liquid forms were also designed for small scale use and experimentation. At present studies on the effects of such supplements are few using EFA and EFB however, the following studies are few that were using CD-CHO and CD-OPTICHO as their basal medium for culturing CHO cells and EFA/EFB as the liquid nutrient feed. Barret *et al*., (2012) evaluated EFB as a feeding regime in their study to increase the antibody titre from CHO cell cultures. They feed 5.6 % of EFB on day 3, 5, 7, 9, 11 and 13 with a total of 40% to the culture of CHO cells in 5 L bioreactor. The results showed that cells grew up to 20 x 10^6 cells/mL with an increase of 110% over the control IgG titre (~3g/L). Similarly, Xiao *et al*., (2014) stated that an increase by two fold of Erythropoietin (EPO) produced by CHO cells cultured with 10% EFB added on day 4 and 7 in a microbioreactor chambers; using CD-OptiCHO as the basal medium. In addition, Baik *et al*., (2015) suggested that by optimizing the bioprocess condition of a CHO cell culture, it may improve the productivity of the cells. I L working volume of CHO-S cell culture were monitored as fed batch culture in a 2 L bioreactor. The basal medium being used was CD-CHO and were fed with 100 mL EFB on day 3, 5, 7, 9 and11 for first run and on day 2, 4, 6, 8 and 10 for the second run. Furthermore Pan *et al*., (2016) did an

experiment to select a suitable chemically defined media for CHO cell fed-batch culture processes. They used two clones of CHO cells from the same parental CHO cells which is a high producer and a low producer that produced the same monoclonal antibody. The study includes testing 4 types of chemically defined media with a combination of three different types of feed giving a total of 12 medium-feed combinations for each cell line. Among the four medium being tested, CD-CHO and CD-OPTICHO were included in the experiment with a combination of both EFA and EFB and were used to culture both low and high producer of cell lines. The high producer cell line cultured in both CD-CHO + EFA/B and CD-OPTICHO + EFA/B produce around 300 mg/L of mAb. However, Pan *et al*. consider it as low because, CHO cells culture in other chemically defined medium and other feed produced higher mAb. Table 5.1 shows the summary for the above mentioned research.

Table 5.1 Research using CHO CD EfficientFeed A (EFA) feeding supplement and CHO CD EfficientFeed B (EFB) feeding supplement.

Cell type and medium used	Culture mode	Feeding regime	Cell density	Product titre	Reference
Proprietary IgG-expressing CHO cells. CD-CHO	5 L bioreactor culture	5.6% EFB on days 3, 5, 7, 9, 11, 13, and 15 (40% total)	20 x 10^6 cells/mL	An increase of 110% over the control IgG titer (~3 g/L).	(Barrett *et al*., 2012)
CHO DG44; CD-OPTICHO	SimCell™ system (microbioreactor chambers with culture volume of ~ 700 µL	10 % (v/v) EFB were added on day 4 and 7	~ 8.5 x 10^6 cells/mL	Increases Erythropoietin (EPO) by twofold	(Xiao *et al*., 2014)
Metabolically engineered CHO-S; CD-CHO	2 L Biostat B bioreactor (Sartorius Stedim, Bohemia, NY) with a 1 L working volume	100 mL of EFB was added on day 3, 5, 7, 9 and 11 for first run and on day 2, 4, 6, 8 and 10 for second run	~ 4 x 10^6 cells/mL	~ 0.08 g/L of bioengineered heparin	(Baik *et al*., 2015)
Chinese Hamster Ovary (CHO) CD-OPTICHO	250 mL shake flasks (VWR) with a 25 mL initial working volume in duplicate	EFA or EFB were fed depend on the consumption rate of glucose	~ 9.5 x 10^6 cells/mL	~ 300 mg/L of IgG1.	(Pan *et al*., 2016)

5.2 Materials and Methods

GS-CHO 42 passage number 7 were cultured in two different commercially available CHO cell media; CD-CHO and CD-OPTICHO which have been kindly provided by ThermoFisher Scientific. Batch cultivations were performed in 5 L bench top bioreactors with a 3 L working volume. The parameters for bioreactor setup were as in section 2.10. The cell numbers were determined by the trypan blue exclusion methods using a haemocytometer (Section 2.4) and the antibody concentrations were determined using ELISA, routinely throughout the culture duration until the cells reached death phase (Section 2.10.2). Feeding protocols were as mentioned in Chapter 2 (Section 2.11). Cultures were terminated once the viability dropped below 60 %.

5.3 Results and Discussion

In fed batch experiments in the bioreactor, the impact of two commercially available culture media with two commercially available liquid nutrient supplements on cell growth and antibody concentrations, as well as the process duration, was evaluated (Fig. 5.1). A summary of the results generated from the fed batch cultures is given in Table 5.2.

Table 5.2 Process relevant data from fed batch culture in 5 L bioreactor working volume of 3 L for GS-CHO 42 cultured in two commercially available medium with two commercially available liquid nutrient supplement.

Medium & feed	**CD-CHO + EFB**	**CD-OPTICHO + EFA**
Peak cell conc. (10^6 cell/mL)	5.15	2.62
IgG conc. (g/L)	0.32	0.09
specific growth rate, μ (h^{-1})	0.028	0.028
Doubling time (h)	24.76	24.76

The viable cell density and viability profiles are shown in Figure 5.1. The growth pattern differed markedly between different media and feeds. GS-CHO cells cultured in CD-CHO media + EFB grew fast during exponential phase and peaked after 7 days at a high cell density (5.15 x 10^6 cells/mL). However, both cells in the different medium and feed; CD-CHO media + EFB and CD-OPTICHO media + EFA have a similar specific growth rate which are 0.028 h^{-1}. Both specific growth rate are tested as not significantly different using the student T-test analysis. The peak cell density of cells cultured in CD-OPTICHO media + EFA were on day 4 which is 2.62 x 10^6 cells/mL before entering stationary phase and death phase after 6 days of culture. The viability dropped below 95% on day 7 in CD-CHO + EFB, while in CD-OPTICHO + EFA viability dropped earlier on day 5 of culture. Doubling time (24.76 h) which is similar for both cells cultured in CD-CHO + EFB and in CD-OPTICHO + EFA are tested as not significantly different using T-test analysis.

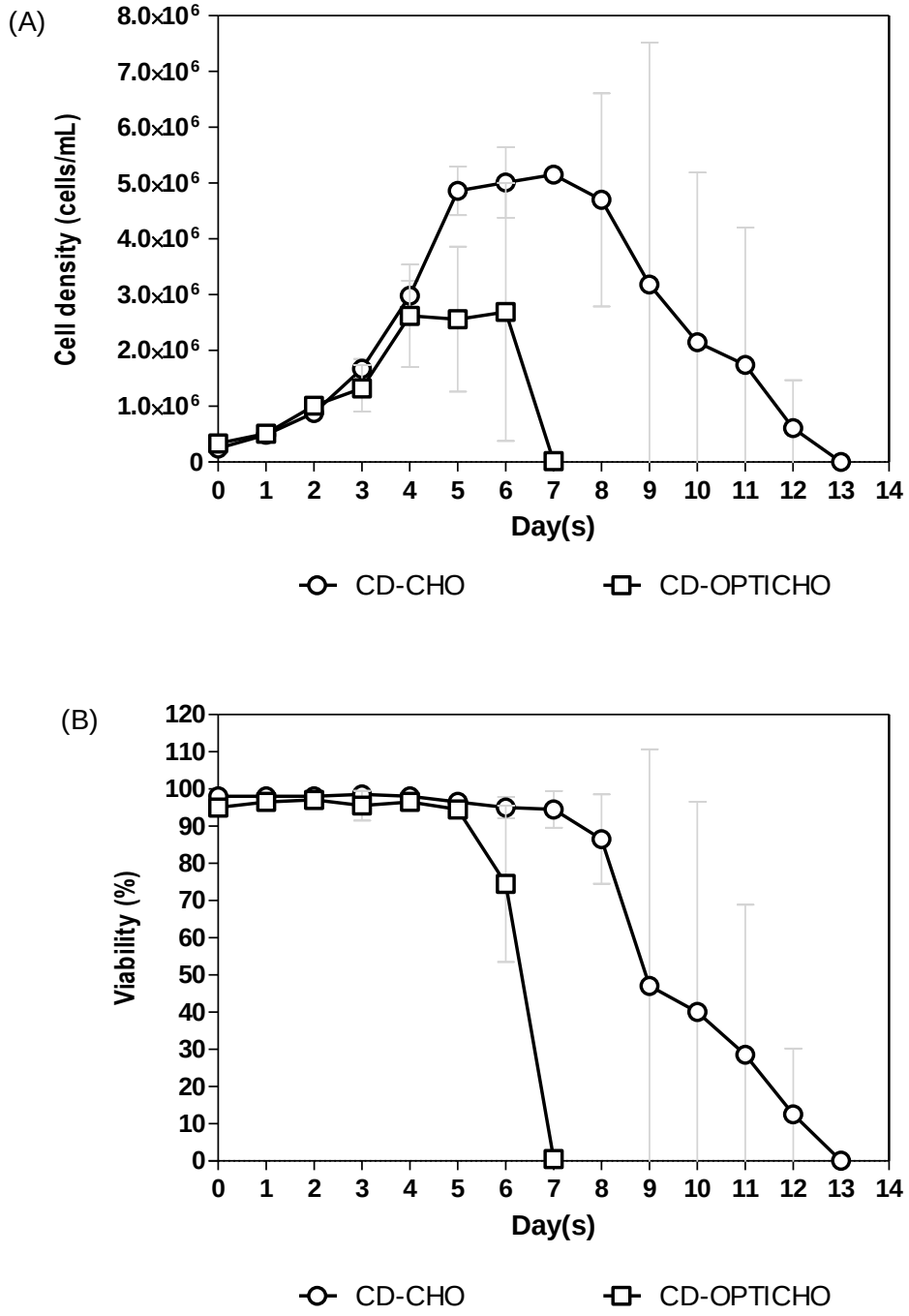


Figure 5.1 Growth of GS-CHO (CHO 42) passage 7 in bioreactor working volume of 3 L cultured in CD-CHO and CD-OPTICHO medium + MSX, 5% CO_2 and 37ºCin fed batch mode. A) The viable cell density profile. B) Viability profile. The data presented are the average values of duplicate samples analysed in duplicate + S.D.

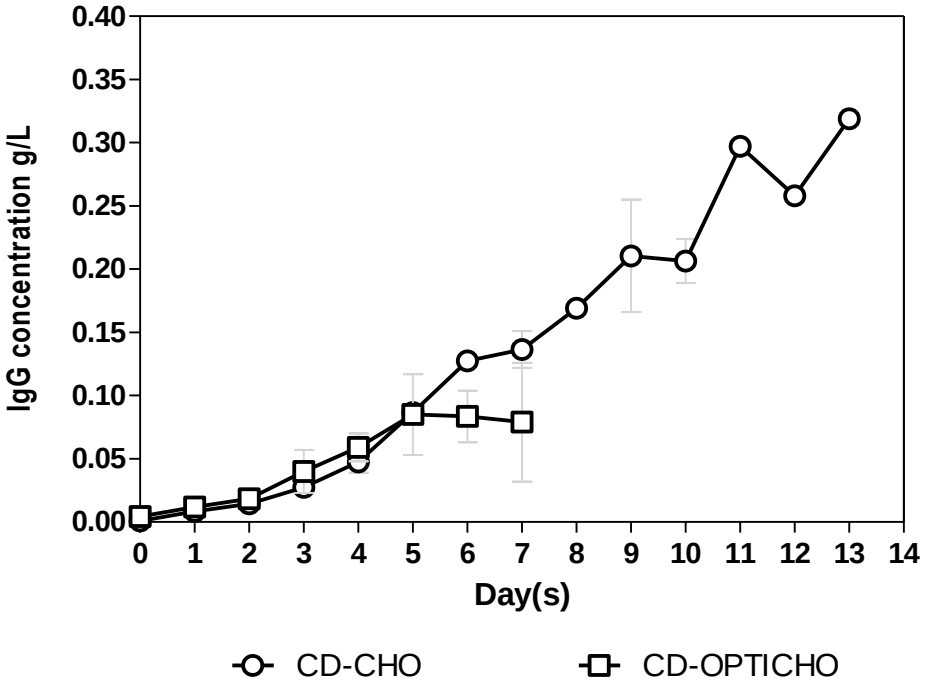


Figure 5.2 MAb concentration of GS-CHO (CHO 42) passage 7 in bioreactor working volume of 3 L cultured in CD-CHO and CD-OPTICHO medium + MSX, 5% CO_2 and 37^{o}Cin fed batch mode. The data presented are the average values of duplicate samples analysed in duplicate + S.D.

Figure 5.2 shows the mAb concentration produced from GS-CHO cultured in two different media with their respective feed supplements (EFA or EFB). IgG concentration in cells cultured in CD-CHO + EFB increased throughout the culture with a cumulative amount of 0.32 g/L on day 13. This is in contrast to IgG produced from GS-CHO cells culture in CD-OPTICHO + EFA where the IgG produced only reached their highest of 0.09 g/L on day 5 before it went down a little bit on day 6 and 7 just as the culture was terminated due to the drop in cell growth and viability.

Table 5.3 Comparison for the process relevant data from batch and fed batch culture in 5 L bioreactor working volume of 3 L for GS-CHO 42.

Medium	**CD-CHO**		**CD-OPTICHO**	
Culture mode	Batch	Fed batch	Batch	Fed batch
Peak cell conc. (10^6 cell/mL)	5.83	5.15	2.48	2.62
Duration of culture before death phase	7	7	7	6
IgG conc. (g/L)	0.37	0.32	0.15	0.09
specific growth rate, μ (h^{-1})	0.032	0.028	0.025	0.028
Doubling time	21.66	24.76	27.73	24.76

The comparison for batch culture and fed batch cultures is shown in Table 5.3. As can be seen from the table, peak cell concentration were high in both batch (5.83 x 10^6 cells/mL) and fed batch (5.15 x 10^6 cells/mL) culture in CD-CHO media, compared to culture in CD-OPTI CHO media with reading of 2.48 x 10^6 and 2.62 x 10^6 cells/mL. However the density of cells are not significantly different among the batch and fed batch culture tested using ANOVA. Results for the peak cell density of cells cultured in CD-CHO + EFB were comparable to a study by Baik *et al*., (2015) where their CHO-S cells were cultured in the same medium and feed (CD-CHO + EFB) reached ~4 x 10^6 cells/mL. However, research by Barrett *et al*., (2012) showed a higher the peak cell density of CHO cells culture in CD-CHO media with EFB which reached almost 20 x 10^6 cells/mL. Then again, Liu *et al*., (2014) cultured different types of CHO cells in CD-CHO media supplemented with various feeds including EFA and EFB. Their maximum viable cell densities were higher compared to this study and ranged from 8.7 to 27.3 x 10^6 cells/mL. In addition, in the study by Carrillo-Cocom *et al*., (2015) using a recombinant CHO cell line cultured in CD-OPTICHO media + EFB they also showed a higher peak cell density than in the present study of 9 x 10^6 cells/mL. Doubling time was not significantly increased in fed batch mode for both culture medium, CD-CHO (batch, 21.66 and fed batch, 24.76) while, CD-OPTICHO (batch 27.73, and fed batch, 24.76).

As can be observed from Figure 5.2, the IgG concentration obtained from CHO cells culture in fed batch mode from two different media and commercially available nutrient supplements which is 0.32 g/L from cell cultured in CD-CHO media + EFB and 0.09 g/L from cell cultured in CD-OPTICHO + EFA. However, as can be seen in Table 5.2, IgG concentration were highest in cell culture via batch mode in CD-CHO

media. Result were also the same for CHO cells culture in CD-OPTICHO medium where batch mode gave higher titre of IgG compared to fed batch mode. These results are at variance compared to other research where it stated that fed batch culture gave higher product quantity. Robitaille *et al*., (2015) in their research of batch and fed batch CHO cell culture, comparing two different culture medium;(Biogro_CHO and PowerCHO-2) observed an increased amount of productivity from cells cultured in fed batch mode. Another example for higher production from fed batch was reported by Altamirano *et al*., (2004) where t-PA produce from CHO cells culture in fed batch mode gave higher concentration compared to batch mode culture. Additionally, Chee Furng Wong *et al*., (2005) also showed an increase in the yields of a recombinant glycoprotein, Inteferon gamma from CHO cells culture in a fed batch mode. The reason for a smaller amount of IgG produced in fed batch culture compared to batch culture in this study were unclear however it may due to the adaptation phase that were required for the cells. This is because, the GS-CHO cells used in this study were being cultured for the first time with the addition of commercial liquid supplementation (EFA and EFB). This may also explain the short duration of fed batch culture of CD-OPTICHO + EFA where it only lasted 6 days before cell death on day 7.

Again in this chapter, the five important extracellular metabolites (glucose, lactate, glutamine, glutamate and ammonia) were monitored and measured. As can be seen from Figure 5.3 (A), glucose concentrations were fluctuating during the culture due to the feed added every two days starting on day 3. As the major carbon source in mammalian cell culture, glucose was consumed constantly throughout the culture. Maximum lactate concentration reached 4.6 g/L on day 10 for cells cultured in CD-CHO + EFB while cells cultured in CD-OPTICHO + EFA reached a lactate

concentration of 2.7 g/L on day 6 (Figure 5.3 (B)). Both these lactate concentrations in the different culture condition did not appear to affect the cell growth performance and productivity. The decreasing pattern of lactate in both culture condition towards the end of the culture may suggest that lactate production has turn into lactate consumption at a low concentration of glucose. This situation has been discussed by Fernandez Martell (2015) where he observed that lactate metabolism switched to net lactate consumption during stationary growth phase in fed batch culture. The peak of lactate concentration is at 4.6 g/L on day 10 for cells cultures in CD-CHO + EFB while cells culture in CD-OPTICHO + EFA reached a peak of 2.7 g/L of lactate on day 6 of culture. Those values are considered as an average amount of lactate in a cell culture where it will not affect the cell growth and productivity. It has been shown in previous study where the amount of lactate did not exceed 4 g/L; 2.61 g/L (Zhang *et al*., 2013), 1.35 g/L (Niu *et al*., 2013) and 4 g/L (Li *et al*., 2012). In addition, study by Liu *et al*., (2014) showed that a lower amount of lactate in their study of different medium and feed supplements which did not exceed 3 g/L provided a less stressful culture environment for cultured cells and allowed sustained high viabilities and extended culture length and also higher mAb titres. Liu *et al*., (2014) studied using CHO-S cells cultured in CD-CHO + EFA and also CD-CHO + EFB. The peak lactate concentration for both were only 1.3 and 1.2 g/L. Another study using CD-CHO + EFB in fed batch bioreactor was by Baik *et al*., (2015)also using CHO-S cell line. Their study also showed a maximal amount of lactate which was lower than 3 g/L. With regard to those study, Pascoe *et al*., (2007) shows a peak of 7 g/L of lactate before the growth of CHO cells ended and expected that the high lactate concentration contribute to a viability decline. Additionally, Dorai (2013), research on proteomic analysis of an antibody

producing GS-CHO cell line shows about 7 g/L by product of lactate in the bioreactor culture resulted in a sharp decline of the viability of cells. As can be seen in this study, a concentration of lactate exceeding 4 g/L starting on day 7 also results in a decline of viability of the GS-CHO (Fig 5.1 (B)). Same goes to the cell growth profile where the cells started to enter its death phase after day 7 when lactate concentration exceeding 4 g/L.

Maximum glutamine concentration was 0.06 g/L in cultures of CD-CHO + EFB and 0.03 g/L in cultures of CD-OPTICHO + EFA (Figure 5.4 (A)). The low level of glutamine in both cultures was expected due to the use of the GS expression system, where GS-CHO cell lines that were used in this study can be grown in a glutamine free media. Therefore, no glutamine were added prior to culture because a GS expression system are capable of producing endogenous glutamine from glutamate and ammonia. The results are comparable to study by Ning (2013) where their analysis results confirmed that no glutamine was presented in the media throughout the whole cell culture process when using GS expression system. Another study is by Zhang *et al*., (2013) where they also found out that glutamine was completely exhausted prior to end of cell proliferation. Ammonia accumulated from 0.02 on day 1 to 0.11 g/L by day 13 in culture of CD-CHO + EFB while it increased from 0.02 on day 1 to 0.08 g/L by day 7 in cultures of CD-OPTICHO + EFA (Figure 5.4 (B)). The reasons for this are not clear and this was a surprising finding for a GS-CHO cell line since its endogenous synthesis of glutamine should result in a reduction of ammonia concentration.

Although the concentration of ammonia are comparable to the study by Niu *et al*., (2013) where the highest concentration of ammonia is approximately 0.03 g/L; while studies by Zhang *et al*. (2013) and Ning (2013) showed that the concentration of ammonia did not exceed 0.6 g/L in both studies, a review by McAtee *et al*., (2014) stated that ammonia concentration of 0.07 - 0.14 g/L is inhibitory to cell growth. Conversely the ammonia levels in cell lines that do not produce GS are much higher which is approximately 1.7 g/L (Han *et al*., 2011).

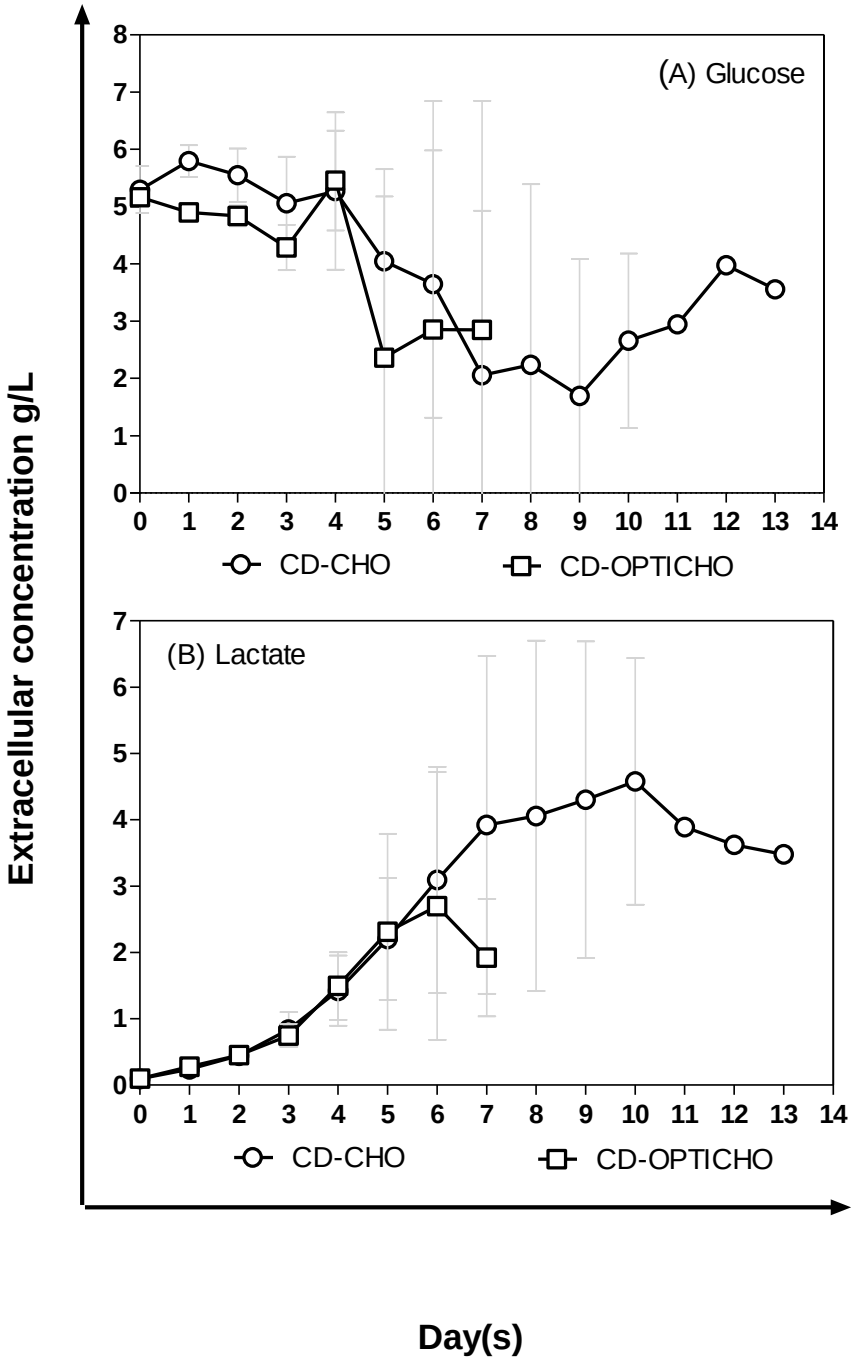


Figure 5.3 Glucose and lactate concentration profiles in the culture of GS-CHO (CHO 42) passage 7 in bioreactor working volume of 3 L cultured in CD-CHO and CD-OPTICHO medium + MSX, 5% CO_2 and 37ºCin fed batch mode. The data presented are the average values of duplicate samples analysed in duplicate + S.D.

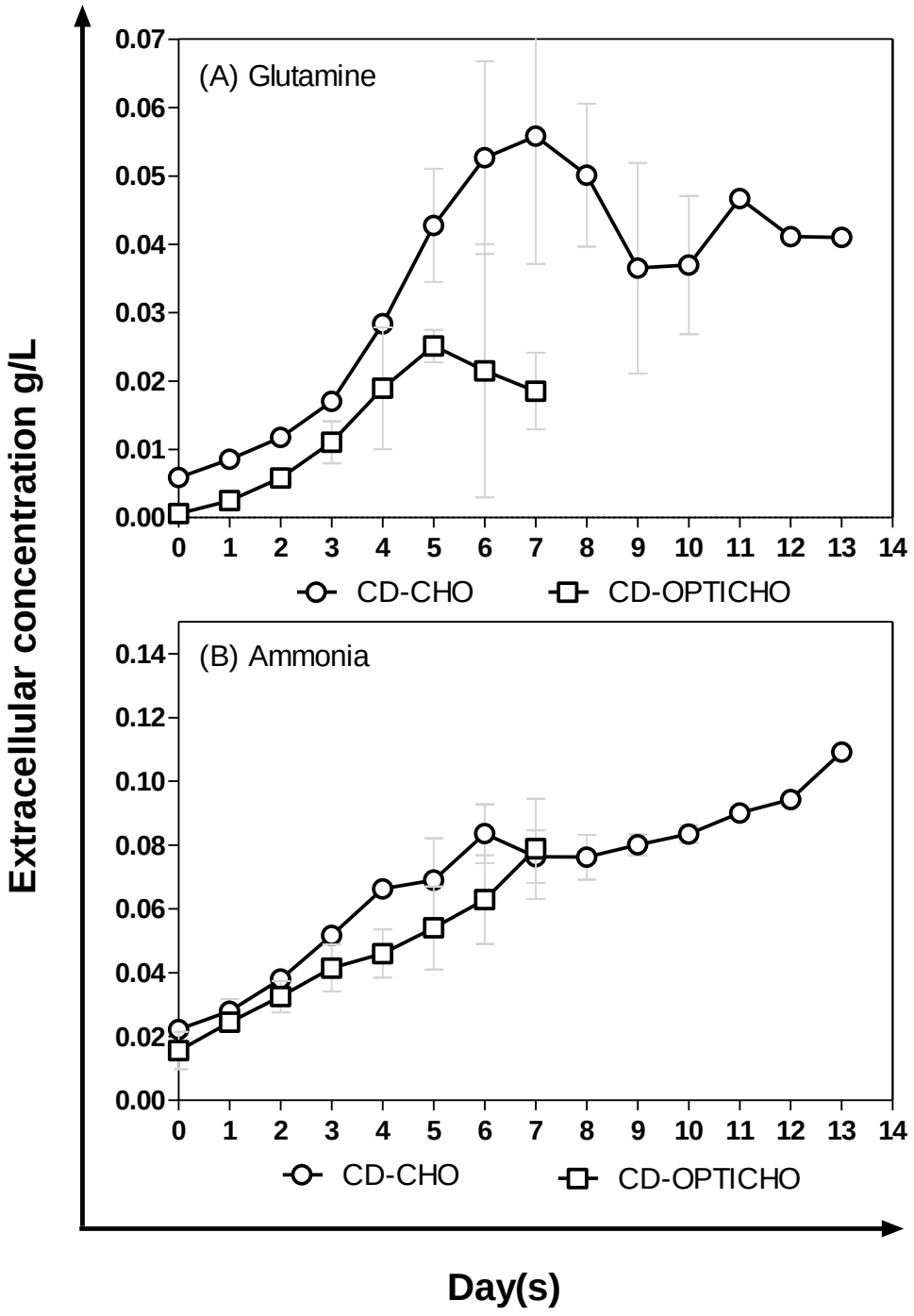


Figure 5.4 Glutamine and ammonia concentration profiles in the culture of GS-CHO (CHO 42) passage 7 in bioreactor working volume of 3 L cultured in CD-CHO and CD-OPTICHO medium + MSX, 5% CO_2 and 37ºCin fed batch mode. The data presented are the average values of duplicate samples analysed in duplicate + S.D.

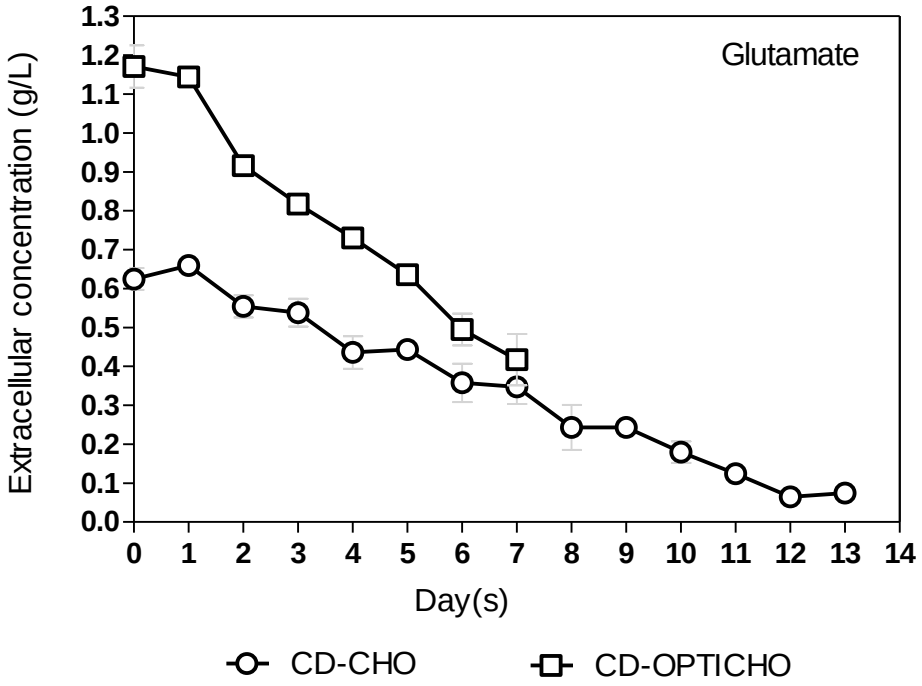


Figure 5.5 Glutamate concentration profiles in the culture of GS-CHO (CHO 42) passage 7 in bioreactor working volume of 3 L cultured in CD-CHO and CD-OPTICHO medium + MSX, 5% CO_2 and 37ºC in fed batch mode. The data presented are the average values of duplicate samples analysed in duplicate + S.D.

Glutamate started with a higher concentration in the culture of CD-OPTICHO + EFA at 1.17 g/L (Figure 5.5) and decreasing to 0.4 on day 7where the culture are in their death phase. While glutamate in CD-CHO + EFB have a lower concentration which is 0.62 g/L and decreasing to 0.07 g/L on day 13. Results shows that it is in agreement with the fact that GS expression system synthesis glutamate to produce glutamine in the culture. In addition, according to the analysis of the initial concentration of glutamate in the fresh feed supplement, the concentration of glutamate in EFA (1.33 g/L) is much higher than in EFB (0.65 g/L).

5.4 Conclusion

In an effort to increase the GS-CHO growth performance and productivity, we tried to use the fed batch culture mode using two different commercially available chemically defined media fed with two types of commercially available feed supplementation. CD-CHO media in particular may serve as a basal media for GS-CHO cells in order to maintain the growth performance and expression of mAb because the GS-CHO cells used in this study were already being cultured and adapted in the CD-CHO media. CD-OPTICHO were used as an alternative because it is known to offer high performance and yield of mAb in a fed batch environment. In addition, two different feed supplementations were used which are EFA and EFB.

The aim of this work was to investigate the effects of different basal media and feeds on cell growth, metabolism, physiology, and mAb production for an industrial CHO cell lines. The GS-CHO cell lines show different metabolic and physiological responses for the different medium-feed combinations that were applied. The best mAb product titer (0.32 g/L) is obtained with CD-CHO basal medium and EFB. For all the tested conditions, the extracellular concentration of the 5 key nutrient which are the glucose, lactate, glutamine, ammonia and glutamate are different for the growth and stationary phase. The result of this study can help to formulate better feed formulation and feeding scheme. However, the actual cause of the cessation for cell growth or death may be multifactorial and unique to each process, which need to be characterized further.

Chapter 6

Metabolomics analysis of industrial CHO cell lines in different culture environment

6.1 Introduction

Chapters 3, 4 and 5 of this thesis have been about the physiology of an industrial CHO cell line, which has been grown in different phases and conditions (different passage number, different cell types, and different media, in shake flask, in bioreactor, batch mode and fed batch mode). Cell line physiology was profoundly affected by most of the process factors based upon cell growth, viability and antibody titre. In the preceding chapters, in addition to the cell characteristics described above, levels of the five key nutrients, glucose, lactate, glutamine, ammonia, and glutamate were monitored throughout the culture process, while other metabolites that may have been present during the culture process and has influenced the process in more subtle ways were not monitored and examined. This chapter will involve the use of metabolomics tools to look into the metabolic profile of industrially relevant GS-CHO cell lines during the culture conditions discussed above. Metabolomics can be used to detect even a small difference between cell lines and culture conditions (Floris *et al.*, 2017; Karst *et al*., 2017; Sellick *et al*., 2015). As can be seen from the previous chapters, there were differences in viable cell numbers and IgG titres between cell passage number, cell types and also modes of culture. Nowadays, knowledge of the amounts of metabolites other than the five major nutrients above that may affect cell growth and productivity are still quite limited. The aim of this chapter is to try to use metabolic profiling to understand better what really happens during the culture process, for example, any depletion or excess of metabolites that may be the reason of the different results of cell physiology studies. In addition, in chapter 4, different media were used to culture the same cell line with identical culture process. However, the physiology

of cells in each medium was significantly different as tested using one way ANOVA (section 4.3). Therefore, it is crucial to know whether any of the nutrients in the different media led to the differences in the physiology of the GS-CHO cells. Based on the findings from Chong *et al.*, (2011), a majority of extracellular metabolites including oxidized glutathione, adenosine-5'-monophosphate (AMP) and guanosine-5'-monophosphate (GMP) were shown to induce apoptosis when introduced to fresh CHO mAb cultures. Chong *et al*., (2011) used a liquid chromatography–mass spectrometry (LC–MS) based metabolomics platform to identify and profile extracellular metabolites in culture media of mammalian cells. Their samples were taken daily from a 4 L fed batch CHO cell culture. Similarly, Selvarasu *et al.*, (2012), stated that a combined metabolomics and *in silico* modelling approach was used to improve understanding of the intracellular mechanisms of CHO fed-batch cultures. The main objective of their analysis was to shortlist metabolites that accumulated in the media over the development of the cultures, as these represented compounds that could be potentially associated with growth-limitation or apoptosis. They shortlisted key metabolites associated with cell growth limitation within the energy, glutathione, and glycerophospholipid pathways that have distinct changes at the exponential stationary transition phase of the cultures. Following in *silico* modelling of CHO cells, they characterized internal metabolic behaviours associated with physiological changes during growth and non-growth phases, thereby allowing them to explore relevant pathways to growth limitation and identify major growth-limiting factors including oxidative stress and depletion of lipid metabolites (Selvarasu *et al*., 2012). Dietmair *et al*., (2012) used a metabolomics approach to analyse differences in metabolite concentrations of CHO cells cultivated in three different media, which

exhibited different growth rates and maximum viable cell densities. They used a combination of HPLC and GC-MS to analyse the intracellular and extracellular metabolites and detected medium specific and time dependent changes in metabolic profiles. These differences in the metabolomes of cultivated cells were apparent in each of three different media (HyQ SFM4CHO, CHO–S–SFMII and CDCHO), as were time dependent changes for each, highlighting the suitability of metabolomics to monitor cell culture processes. Multivariate analysis assisted in the identification of several features that may be directly related to these differences in growth rate (e.g., CTP, UDP-glcA), establishing a rational basis for further investigation of the differences in medium performance. Karst *et al*., (2017) investigate the physiologic state of industrial CHO cell line when cultured in perfusion mode using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS approach. They have two objectives in their study, where the first one is to assess the stability of cellular physiology in a single run during 26 days and the second objective is to investigate the difference between three steady state processes with varying viable cell density (20, 60 and 40 x 10^6 cells/mL). By tandem MS (MS/MS) and high resolution Fourier transform ion cyclotron resonance (FT-ICR) MS, 76 metabolites were identified. Clear clustering according to cell density was observed by principal component analysis, indicating steady-state related metabolic profiles. More precisely, varying levels of nucleotides, nucleotide sugar, and lipid precursors explained most of the variance between the different cell density set points. The developed experimental and data treatment procedure resulted in a reliable and meaningful connection of macroscopic variables (specific growth rate, viability) to intracellular metabolic processes; therefore, this makes it a suitable method for the at-line correction of cell culture

performance during process development and operation. Steinhoff *et al*., (2016) also described (MALDI-MS)- based on a microarray for mass spectrometry (MAMS) technology to rapidly monitor a broad panel of analytes, including metabolites and proteins directly from the unpurified cell supernatant or from host cell culture lysates in batch and perfusion cultures. Intracellular mass profiles for metabolites and proteins can be used to track cellular growth and cell productivity. From the result, more than 100 mass features were detected and 61 were identified by accurate mass using online databases (metlin.scripps.edu, hmdb.ca) and partially by MS/MS experiments. The identified metabolites were mainly phosphorylated nucleotides. The study concluded that though the number of detectable metabolites is limited in MALDI mass spectrometry, it is specifically sensitive towards the detection of a high number of phosphorylated species which are difficult to monitor to this extent using HPLC–MS on the other hand. In addition Floris *et al*., (2017) used an untargeted LC-MS/MS platform to monitor dissimilarities in CHO cell culture media upon exposure to high temperature short time (HTST) treatment, a commonly used viral clearance upstream strategy. Chemically defined media formulations, which comprise only components of traceable origin, can minimize the risk of contamination but not completely remove it. Conversely, supplementation with undefined components, such as plant hydrolysates, to obtain high protein titres which are sourced from unsterile environments, which therefore increases the risk of contamination via introduction of viral species. Thus, upstream barriers for viral deactivation, such as HTST treatment, have been widely used. Nevertheless the quality of media can deteriorate upon treatment at high temperature. Multivariate data analysis techniques were applied to identify relationships between HTST treatment and medium quality alterations. CHO culture

experiments were performed using both treated and untreated formulations to evaluate the effects of the treatment on cell growth performance. Unsupervised chemometric analysis of LC-MS/MS data, revealed clear separation of HTST-treated samples from untreated counterparts as observed from analysis of principal components. Treated samples were inspected for potential "browning" which could develop as a consequence of Maillard product formation due to the reaction of medium constituents, such as amino acids and carbohydrates, at elevated temperatures. Floris *et al*., (2017) study which were among seven types of different medium (3 of chemically defined media and four of soy hydrolysates media). Degradation of amino acid which formed organic acids were found to be increase in CD media upon heat treatment. This include leucine which is from the reaction of D-glucose and L-leucine. Amino acid (cysteine and methionine) or vitamin (thiamine) were both potentially be the sourced of sulphur presents in the CD media after treatment of HTST. Other feature of interest found in the CD medium are nonate, a derivative of succinic acid and glutarylcarnitine, a product obtained from the reaction of glutaric acid with L-carnitine. On the other hand, soy hydrolysate medium analysis upon HTST treatment showed an increase amount of 2-aminoacetophenine, a degradation product of tryptophan and a decrease amount of the dipeptide lysyl-leucine. Other identified interesting features are heterocyclic compounds (oxazoles, pyrazines, and pyrrolidines), a phenolic glycoside, niazimicin A, a benzopyran derivative, acids, aldehydes, and furans. Upon the HTST treatment, hydrolysates medium was more profoundly to be affected compared to CD medium due to the increase level of carbohydrates and amino acids from the late stage Maillard degradants (the product of the process of degradation). However, Floris *et al*., (2017) concluded that the formed Maillard products were not found to be toxic toward CHO

cells as no detrimental effects on cell culture performance were observed, suggesting that key nutrients required for cellular growth were not impacted by the heat treatment.

In this chapter application of an LC-MS based metabolomics analytical strategy to observe different metabolite distribution that may influence low viability, cell density, and the IgG titre in a certain culture conditions is described.

6.2 Methods

1 x 10^7 cells were obtained daily from each culture condition; described in section 2.9 for shake flask, section 2.10 for batch culture and section 2.11 for fed batch culture beginning at 24 hour. The cells were quenched and extracted as described in section 2.13.1 and Figure 2.9. Then the samples were analysed using LC-MS as described in section 2.13.2. After that, the data were processed as in section 2.13.3. Briefly, the process for metabolomics sample processing is shown in Figure 6.1. Samples from different scales of culture, shake flasks or bioreactor, different media (CD-CHO, CD-OPTICHO and Dynamis) and different culture modes (batch or fed batch) were collected daily. After that, samples underwent the quenching process to stop cell metabolism and allow medium removal; while an extraction process recovered the metabolites from the cells. The extracted samples were subsequently injected in triplicate into the Exactive Orbitrap MS (Therno Scientific). Xcalibur and MZmine software were used to pre-process the resultant MS data and putative metabolite identities were first assigned through mass comparison with online databases KEGG and HMDB. Statistical analysis with Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were perform using SIMCA-P+ version 14.1.

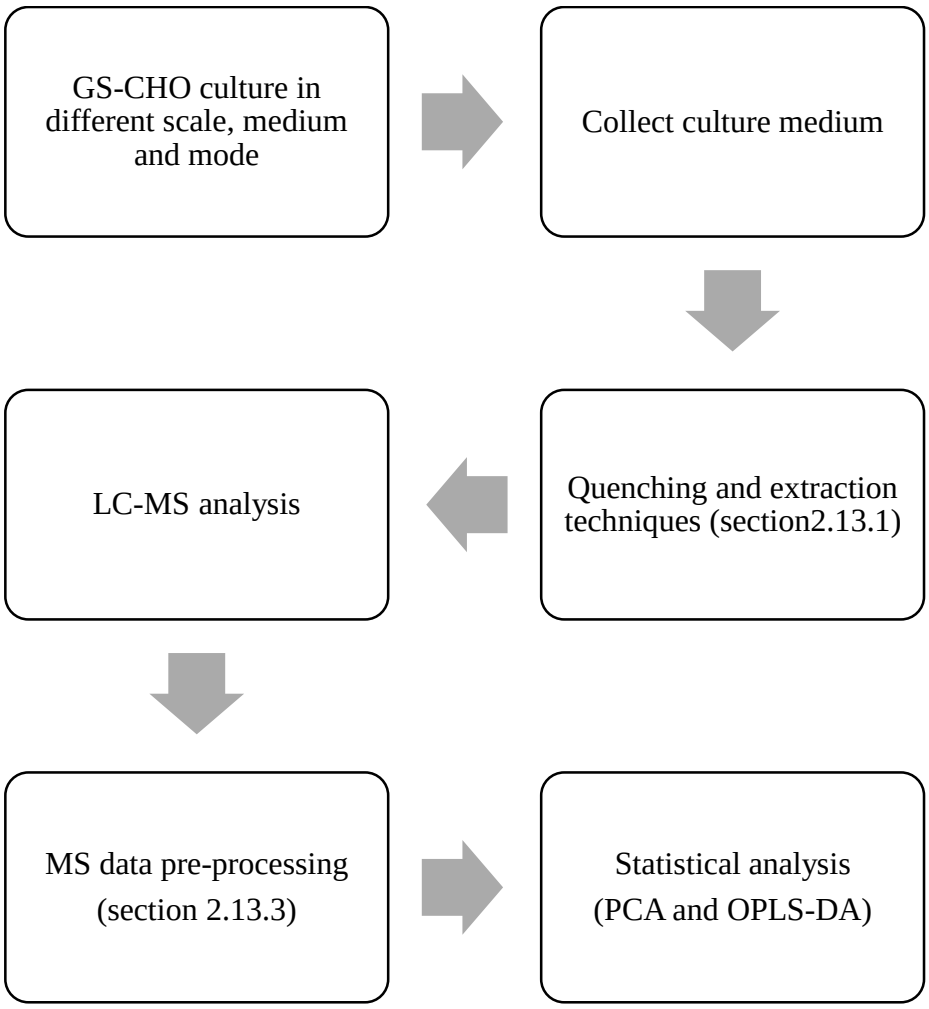


Figure 6.1 Metabolomics sample processing adopted for the analysis of GS-CHO cells culture.

6.3 Results and discussions

An intracellular metabolic profiling study was conducted on the GS-CHO cell lines cultured under a range of conditions to identify important metabolic pathways that are closely associated with growth limitation and product titre.

The metabolites of interest were putatively identified by first matching their observed high resolution masses with compounds found in KEGG and HMDB databases. The identities of some metabolites could be confirmed using the LC/LTQ-Orbitrap system by comparing LC elution times and MS^2 fragment ions with standards. However a large number of metabolites detected using LC-MS could not be identified or only uncertainly identified using the databases from KEGG and HMDB. This is a common downside in metabolomics as mentioned by Dietmair *et al*., (2012) and many others. In the same way, Gika *et al.*, (2014) discussed problems and the limitations of LC-MS based metabolomics. They stated that metabolite identification remains a major drawback in current LC-MS metabolomics. The process is not straightforward as the number of putative identities can be huge and varied. In addition, in the present research, instrument availability and time-consuming post process analysis limited the amount of identification possible, thus only comparisons of putative metabolites were using the KEGG and HMDB databases.

Overall, this generated a relatively narrow sample of the rich metabolomes in these cell line cultures. If time had permitted, it would have been possible to carry out more extensive identification of the specific metabolites present using the approaches discussed above. Nevertheless, the research may be a useful step in looking into the interesting metabolites distributed in each of the following plots to look into the

metabolic profile of the industrial GS-CHO cell lines in order to improve the production of monoclonal antibody which are commonly used in the treatment of many previously difficult to treat diseases and conditions.

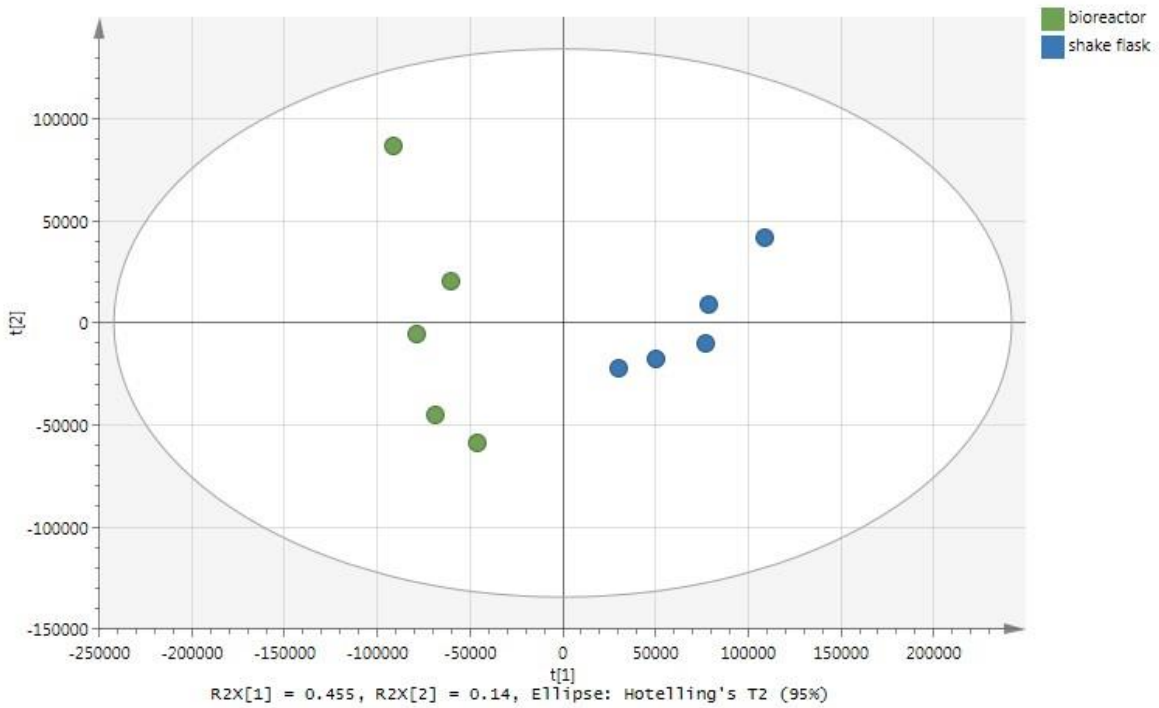
6.3.1 Multivariate analysis for shake flask cultures and bioreactor cultures

Multivariate analysis was performed on the GS-CHO 42 cell lines cultured in different conditions to obtain the metabolic profile of each culture condition and to evaluate the relationship between metabolites in the GS-CHO cell lines from various conditions. The unsupervised PCA method is the most commonly used multivariate technique to summarize a data set (Richardson *et al*., 2015; Tescione *et al*., 2015; Floris *et al*., 2017; Karst *et al*., 2017 and Sokolov *et al*., 2017). It involves the transformation of original set of correlated variables to uncorrelated variables, called principal components. The results are displayed as scores plots indicating the scatter of the samples, which indicate similar metabolome compositions when clustered together and compositionally different metabolomes when dispersed.

The PCA scores plot showed strong separation between the GS-CHO cells (Figure 6.2 A) cultured in shake flask and in bioreactor in terms of their metabolic profiles. The grouping for different scales of culture can be clearly seen (Figure 6.2 A). Results can be also be summarized by looking at the loadings plots (Figure 6.2 B). The loadings plot shows how the original variables load or contribute to the component. Variables which have little contribution to a direction have almost zero weight in the loadings while strongly correlated variables will have approximately the same weight value

when they are positively correlated (Bro and Smilde, 2014). Therefore they will appear near each other in a loadings plot while negatively correlated variables will appear diagonally opposite each other. The loadings plot, (Figure 6.2 B) relate the influence of each considered feature to the variation of the respective principal component. The features explaining most of the variance in the principal components are located at the extremes of the loadings variables similar to the clusters in the scores plot (Figure 6.2 A). Based on the scores plot, different metabolites appeared to be separating at different types of culture scale (shake flasks and bioreactors). Therefore, it may be that samples from the upper right quadrants are metabolites present in the shake flask cultures while the lower left quadrants consists samples of metabolites present in bioreactor cultures. Table 6.1 shows the discriminating metabolites extracted from PCA loadings plot of GS-CHO in shake flask and bioreactor samples.

(A)



(B)

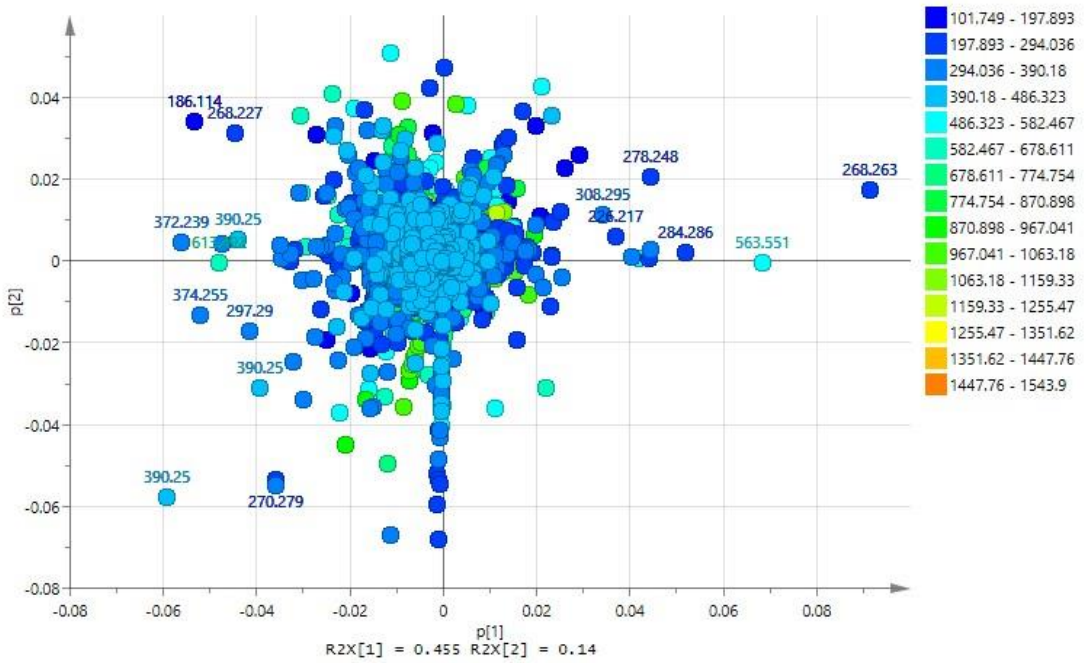


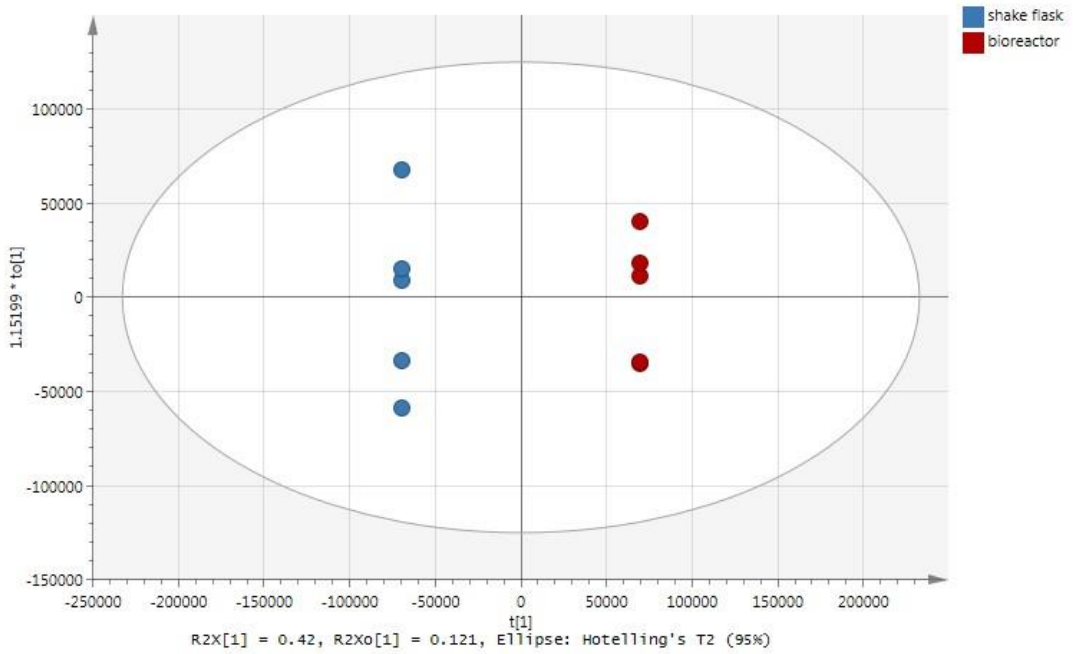
Figure 6.2: (A) The PCA scores scatter plot of the GS-CHO cell cultures showed clear separation between cultures in shake flask and culture in bioreactor. (R2(Y) = 0.70; Q2(Y) = 0.30; (B) The loadings plot showed the distribution of the metabolites produced. The unique metabolites found in all extract listed on **Table 6.1**.

Table 6.1 Discriminating metabolites extracted from PCA loadings plot of GS-CHO (Figure 6.2) in shake flask and bioreactor samples.

Bioreactor						
Primary ID	***m/z***	**RT (min)**	**Peak Area (b3-d9-xq)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LipidMaps/KEGG database**
N_8091	186.1140	5.61	3.09E+05	187.1210	$C_9H_{17}NO_3$	8-amino-7-oxononanoate
N_8106	374.2550	15.08	4.60E+07	375.2623	$C_{19}H_{37}NO_6$ [a]	No hits
N_9024	390.2500	16.08	1.44E+07	391.2573	$C_{19}H_{37}NO_7$ [a]	No hits
N_3465	390.2500	16.32	3.71E+07	391.2571	$C_{19}H_{37}NO_7$ [a]	No hits
N_8123	372.2390	16.63	5.22E+07	373.2467	$C_{19}H_{35}NO_6$	dodecanedioylcarnitine
P_35104	268.2270	17.30	4.37E+07	267.2198	$C_{16}H_{29}NO_2$ [b]	(4E,8E,10E-d16:3) sphingosine
N_3692	358.2600	20.32	3.83E+07	359.2671	$C_{19}H_{37}NO_5$	2-hydroxylauroylcarnitine
P_35102	613.4920	20.92	4.23E+07	612.4848	$C_{34}H_{60}N_8O_2$	No hits
P_46567	297.2900	28.30	2.42E+06	296.2827	$C_{18}H_{36}N_2O$ [b]	1-deoxy-18-amino-(4E,6E,d18:2) sphinganine
P_44175	270.2790	29.54	4.86E+06	269.2718	$C_{17}H_{35}NO$ [b]	1-deoxy-(4E,d17:1) sphinganine
Shake flask						
Primary ID	***m/z***	**RT (min)**	**Peak Area (sf-42-q1)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LipidMaps/KEGG database**
P_35137	278.2480	23.36	4.33E+07	277.2410	$C_{18}H_{31}NO$	1-deoxy-(5E,9Z,12Z,15Z,d18:4) sphinganine
P_37681	226.2165	20.94	3.07E+07	225.2092	$C_{14}H_{27}NO$	1-deoxy-(4E,6E,d14:2) sphinganine
P_35064	268.2634	26.74	1.81E+08	267.2561	$C_{17}H_{33}NO$	1-deoxy-(4E,6E,d17:2) sphinganine
P_48306	284.2855	28.60	6.30E+07	283.2782	No prediction	No hits
P_35072	563.5514	28.65	8.09E+07	562.5442	$C_{36}H_{70}N_2O_2$ [b]	No hits
P_38150	308.2946	29.03	2.08E+07	307.2873	$C_{20}H_{37}NO$	1-deoxy-(4E,8E,10E-d20:3) sphinganine
						[a] carnitine congener [b] sphingolipid congener

Another statistical method that were used in this study is the OPLS-DA. In this technique, class information (shake flask and bioreactor in the current study) is used as a categorical Y variable. The technique is used to sharpen the difference between groups of observation and subsequently understand the variables that are responsible for the separation between groups. All process and product quality data were regarded as X data and OPLS-DA will give superior results whenever significant within-class differences exist. Figure 6.3 A shows a supervised multivariate OPLS-DA analysis exhibiting 2 clear classes – a shake flask group, clustered in the same group (highlighted in blue), and a bioreactor group (highlight in red) clustered in the other group. An S plot is an easy way to visualize an OPLS-DA classification model. It has mainly been used to filter out putative metabolites from metabolomics data. In the S plot, both magnitude (intensity) and reliability is visualized and the list of potential metabolites which are statistically significant can be obtained. The S-plot in Figure 6.3 B shows the metabolites at the process end point of the plot; shake flask (lower left) and bioreactor groups (upper right).

(A)



(B)

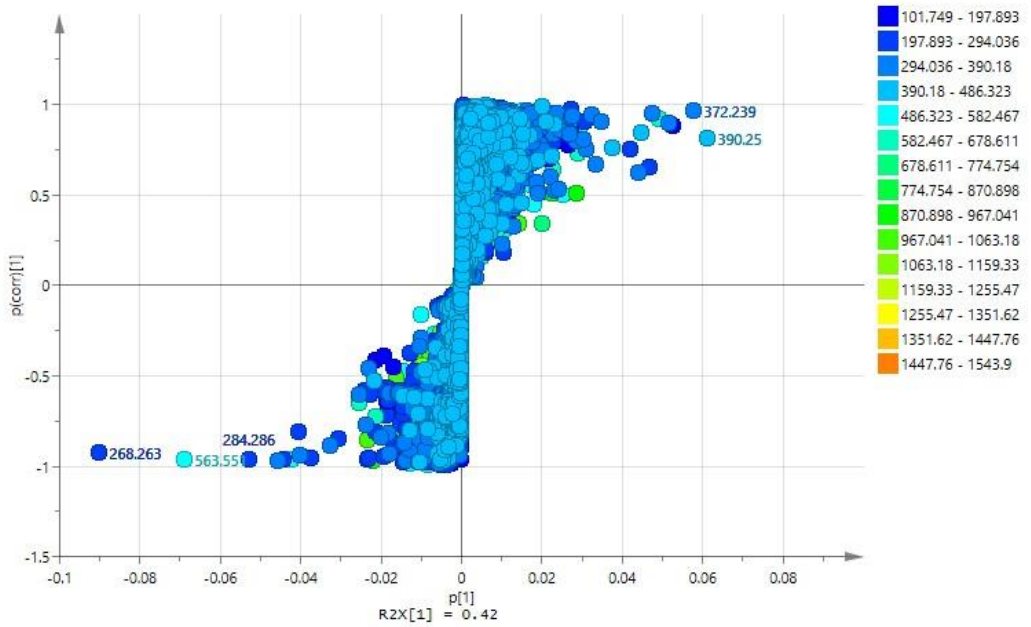


Figure 6.3 (A) OPLS-DA scores plot analysis showed a distinctive separation between the shake flask and bioreactor culture (R2(Y) =1.0; Q2(Y) =0.95); where the variation between the groups is 42 % and variation within the groups is 12.1 %. (B) OPLS-DA S-plot exhibited the metabolites at the end-point for each group. The unique metabolites found in all extract listed on **Table 6.1**.

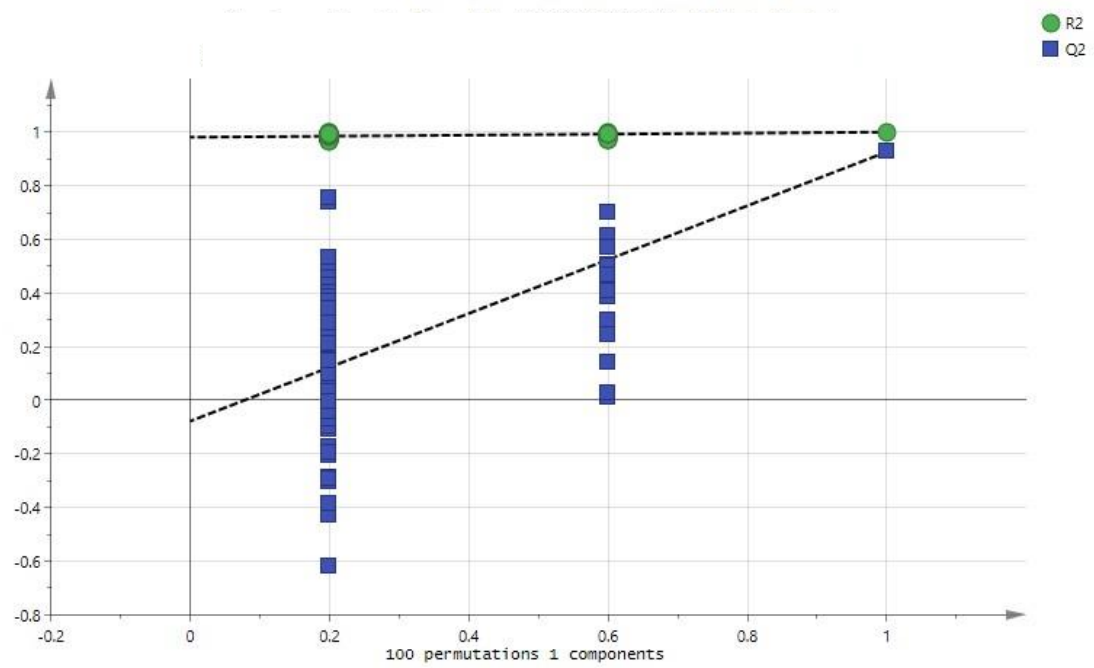


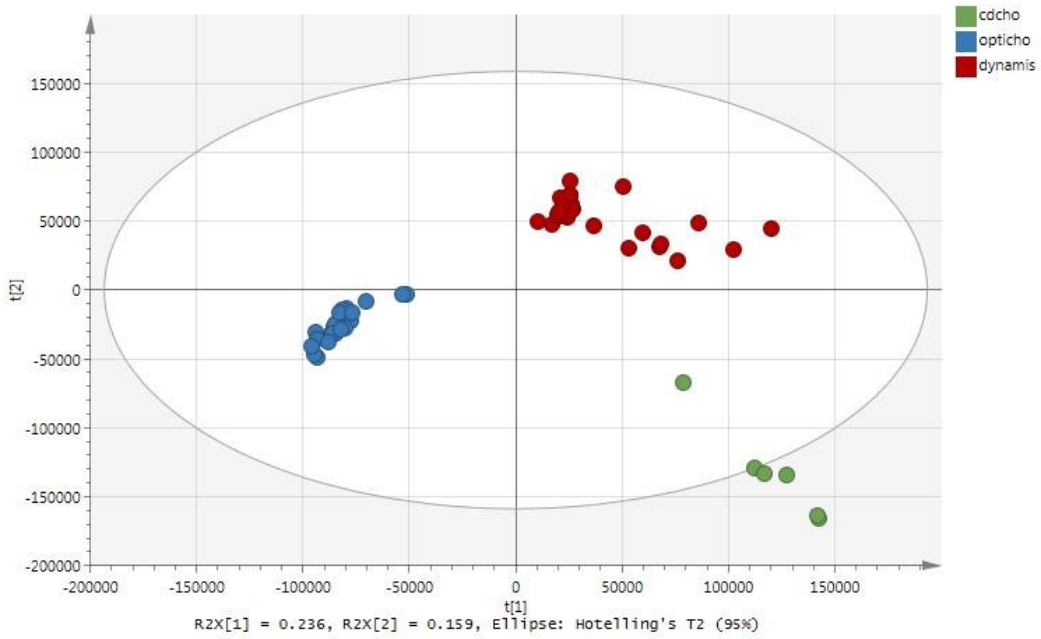
Figure 6.4: Permutation test of shake flask and bioreactor culture generated a Q2Y = - 0.0779 validated the model.

Ahuja *et al*., (2015) used multivariate analysis (MVA) to investigate differences in three scales of culture; shake flasks, 3 L bioreactor and 15 000 L bioreactor. From different types of MVA methods including PCA and OPLS-DA, they found that shake flask cultures have similarities to 15 000 L bioreactor whereas significant differences were found between those two culture scales and the 3 L bioreactor culture proving that shake flasks mimicked 15 000 L runs better when compared with 3 L cultures. Ahuja's result were similar to the findings of the present study where the PCA scores plot of different scale of culture (shake flask and bioreactor, Figure 6.2 A) showed distinctive separation between the two groups of culture scales. In addition, OPLS-DA scores plot (Figure 6.3 A) confirmed the clear groupings of the two culture scales metabolomes and showed that differences in metabolic profiles existed between the 2 scales.

6.3.2 Multivariate analysis for batch cultures of bioreactor with different medium

In Chapter 4 of this thesis, the physiology of GS-CHO cells cultured in batch mode of bioreactor in three commercially available culture medium was discussed. Based on the findings, it was proven that different media resulted in different growth profiles (cell density and viability) as well as antibody production. In order to get more information into what may happen during the culture, and to assess if different media produced different metabolites, a metabolomics study was carried out on the samples taken each day from the bioreactors cultures. Figure 6.5 A shows an unsupervised scores plot analysis for the samples from GS-CHO cells cultured in three different commercially available chemically defined media (CD-CHO, CD-OPTICHO and Dynamis); cultured in a batch mode bioreactor. A distinct separation between the three different media was apparent, with three clear groups in the scores plot. In addition, Figure 6.5 B shows the unsupervised PCA loadings plot where, when compared with the scores plot, we can observe the different of metabolic profiles of each medium. The upper right quadrant represents metabolome from GS-CHO cells cultured in Dynamis medium, the lower right quadrant contains the metabolome that appear in the culture of CD-CHO medium while the lower left quadrant represents metabolome from the GS-CHO cells cultured in CD-OPTICHO medium. The clear separation shows that there are distinctly different metabolic profiles are associated with each of the media.

(A)



(B)

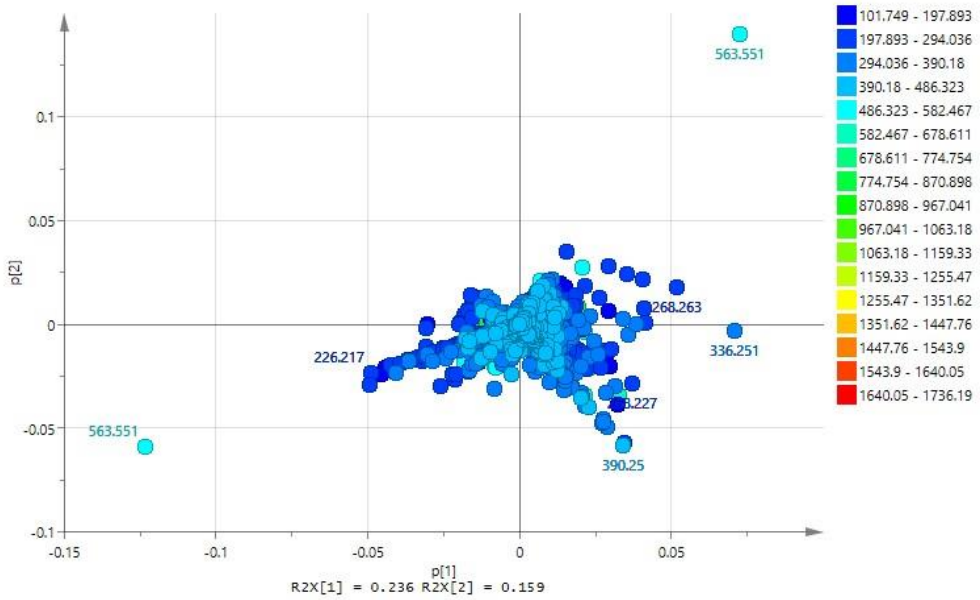


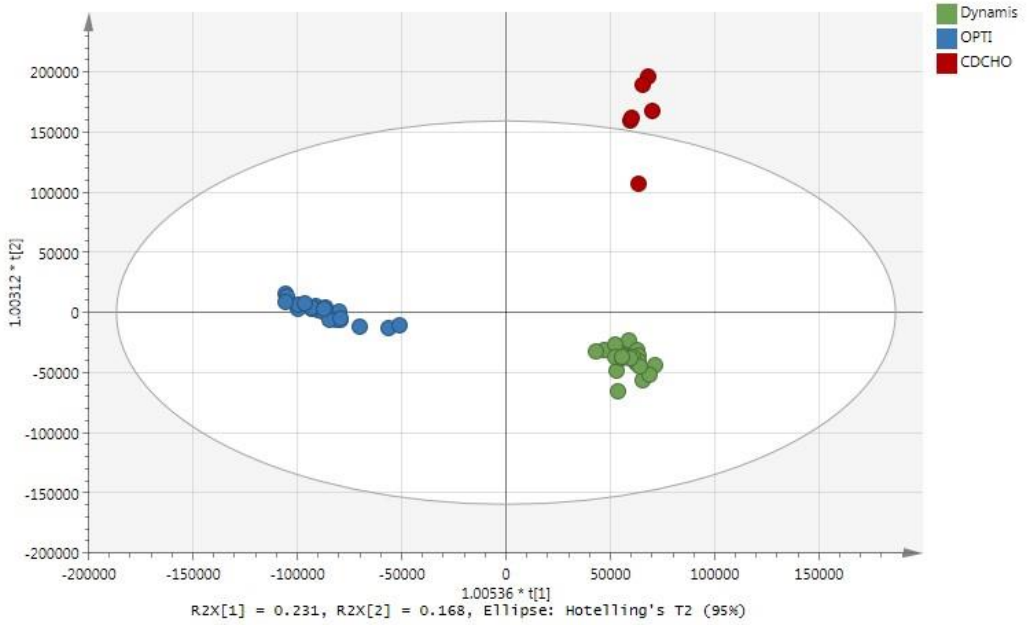
Figure 6.5: PCA analysis of GS-CHO cells cultured in three different medium (CD-CHO, CD-OPTICHO and Dynamis) in batch mode. (A) The PCA scores plot of the GS-CHO cells showed distinctive separation between different medium; (R2(Y) = 0.66; Q2(Y) = 0.40; (B) PCA loadings plot showed the end metabolites produced. The unique metabolites found in all extract listed on **Table 6.2**.

Table 6.2 Discriminating metabolites extracted from PCA loadings plot Figure 6.5 (B) in CD-CHO, CD-OPTICHO and Dynamis media.

CD-CHO						
Primary ID	***m/z***	**RT (min)**	**Peak Area (b1-d5-xq)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LipidMaps /KEGG database**
N_3465	390.2499	16.32	5.26E+07	391.2571	$C_{19}H_{37}NO_7$ [a]	No hits
P_35104	268.2271	17.30	298E+07	267.2198	$C_{16}H_{29}NO_2$ [b]	(4E,8E,10E-d16:3) sphingosine
CD-OPTICHO						
Primary ID	***m/z***	**RT (min)**	**Peak Area (optiB1D6)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LipidMaps /KEGG database**
P_42077	226.2165	22.87	3.62E+07	225.2092	$C_{14}H_{27}NO$	1-deoxy-(4E,6E,d14:2) sphinganine
P_42074	563.5506	30.46	2.14E+08	562.5433	$C_{36}H_{70}N_2O_2$ [b]	No hits
Dynamis						
Primary ID	***m/z***	**RT (min)**	**Peak Area (D1D0)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LipidMaps /KEGG database**
P_35091	336.2507	20.42	1.52E+08	335.2434	$C_{16}H_{29}N_7O$	No hits
P_35064	268.2634	26.74	2.32E+07	267.2561	$C_{17}H_{33}NO$	1-deoxy-(4E,6E,d17:2) sphinganine
P_35072	563.5514	28.65	1.50E+08	562.5442	$C_{36}H_{70}N_2O_2$ [b]	No hits
						[a] carnitine congener [b] sphingolipid congener

Furthermore, OPLS-DA was performed between the three chemically defined media. Figure 6.6 A shows the supervised OPLS-DA for GS-CHO cells cultured in CD-CHO, CD-OPTICHO and Dynamis medium, while Figure 6.6 B shows the S-plot with metabolome at the end-point which have been produced in both the CD-CHO and CD-OPTICHO media. A good separation between CD-CHO and CD-OPTICHO medium was observed (Figure 6.5 A). The end point of the S plot in Figure 6.5 B shows the metabolome for each group (CD-CHO and CD-OPTICHO medium). Clearly different metabolites were produced in the different media and the metabolic differences noted may well be associated with the differing physiology of the cells as discussed in Chapter 4. This also indicates that there were significant differences in the metabolic profiles of cultures in the different media. Comparison between CD-CHO medium with Dynamis medium and CD-OPTICHO medium with Dynamis medium were also done using the OPLS-DA (Figure 6.8 A and B). The scores plot showed good separation between the groups. While S-plot depicted a different group of metabolome for each medium appear at the end of each point of the S plot.

(A)



(B)

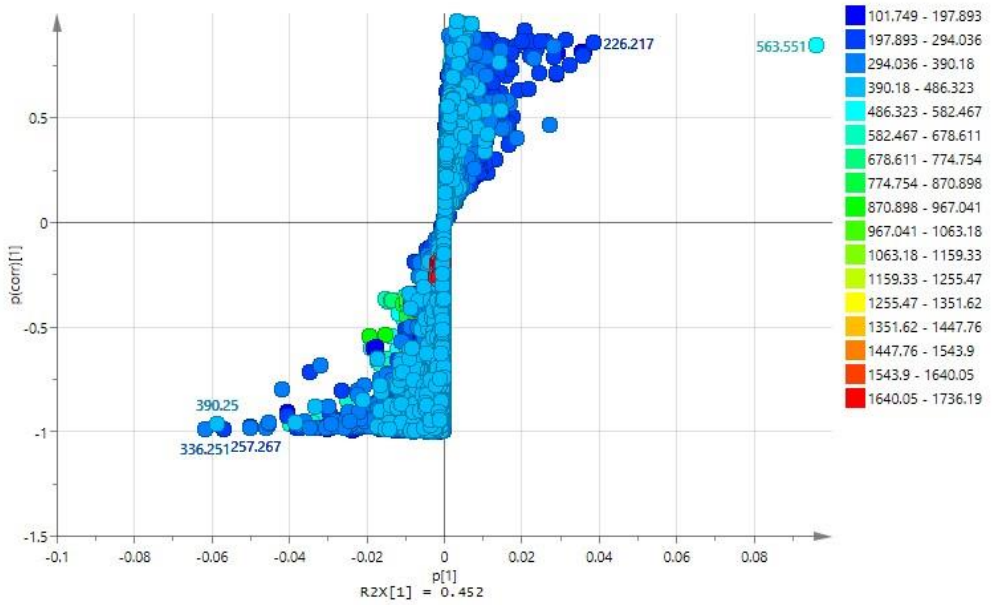
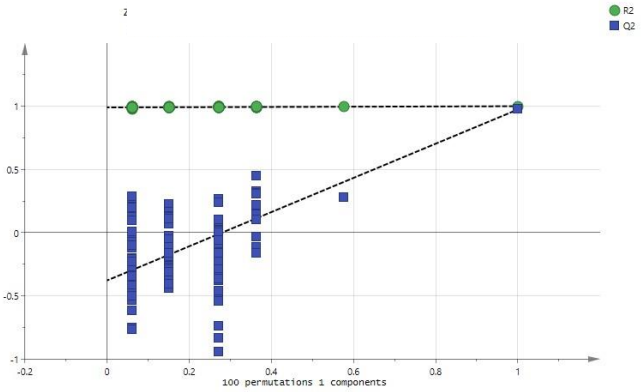
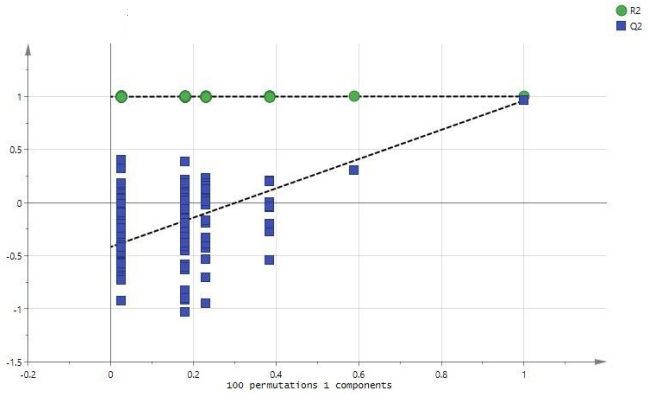


Figure 6.6: (A) OPLS-DA analysis on GS-CHO cells cultured in different medium (CD-CHO, CD-OPTICHO and Dynamis) in batch mode. (R2(Y) =0.97; Q2(Y) =0.97); where the variation between the groups is 23.1 % and variation within the groups is 16.8 %. (B) S-plot exhibited metabolites on both CD-CHO (lower left) and CD-OPTICHO (upper right) groups (R2(Y) =1; Q2(Y) =0.97). The unique metabolites found in all extract listed on **Table 6.4**.

(A)



(B)



(C)

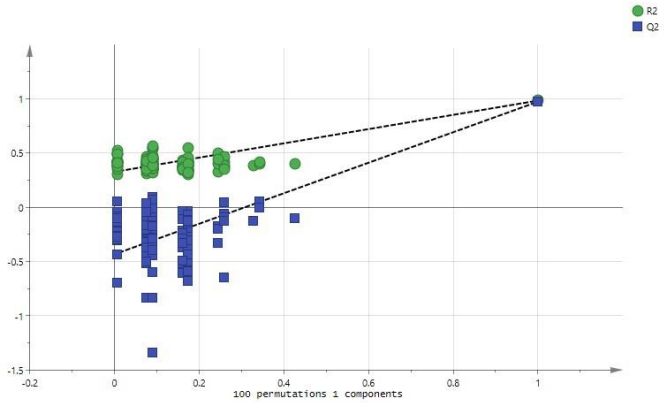
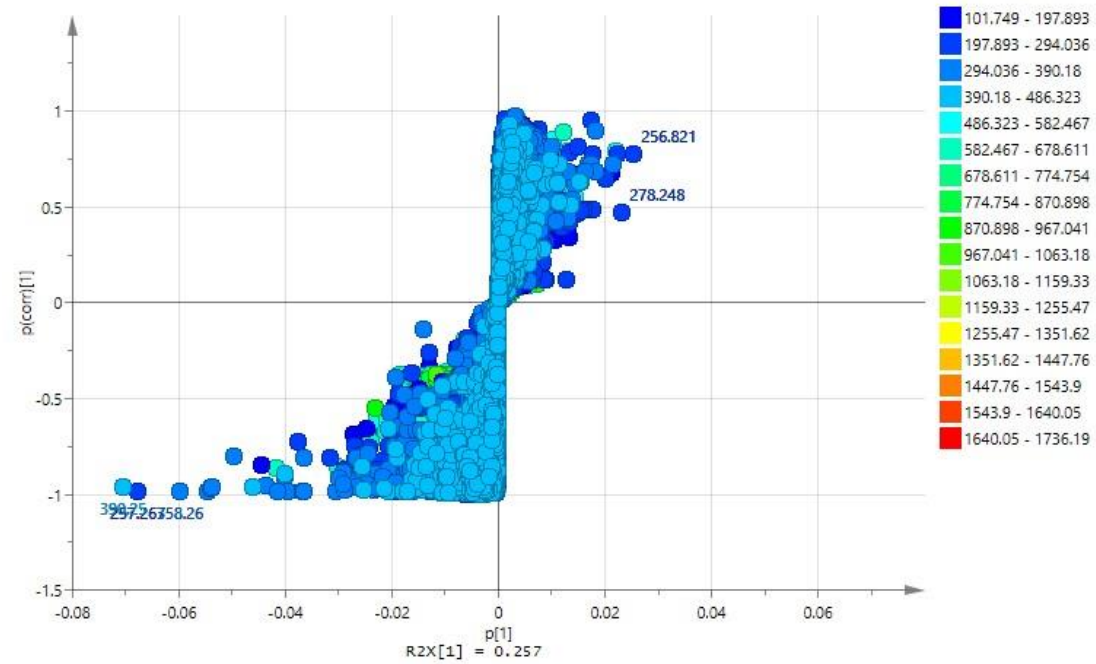


Figure 6.7: (A) Permutation test of CD-CHO and CD-OPTICHO generated a Q2Y = - 0.378 validated the model. (B) Permutation test of CD-CHO and Dynamis generated a Q2Y = - 0.418 validated the model. (C) Permutation test of CD-OPTICHO and Dynamis generated a Q2Y = - 0.4338 validated the model.

Table 6.4 Discriminating metabolites extracted from S-plot (Figure 6.5 (B)) in CD-CHO and CD-OPTICHO culture samples.

CD-CHO							
Primary ID	***m/z***	**P values P ≤ 0.05**	**RT (min)**	**Peak Area (b1-d9-xq)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LIPIDMAPS/KEGG database**
N_3465	390.2499	0.9195	16.32	8.63E+07	391.2571	$C_{19}H_{37}NO_7$ [a]	No hits
P_35091	336.2507	0.9881	20.42	9.02E+07	335.2434	$C_{16}H_{29}N_7O$	No hits
P_37677	257.2667	0.9709	29.02	5.96E+07	256.2594	No prediction	No hits
CD-OPTICHO							
Primary ID	***m/z***	**P values P ≤ 0.05**	**RT (min)**	**Peak Area (optiB2D2)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LIPIDMAPS/KEGG database**
P_42077	226.2165	0.8800	22.87	2.33E+05	225.2092	$C_{14}H_{27}NO$	1-deoxy-(4E,6E,d14:2) sphinganine
							[a] carnitine congener [b] sphingolipid congener

(A)



(B)

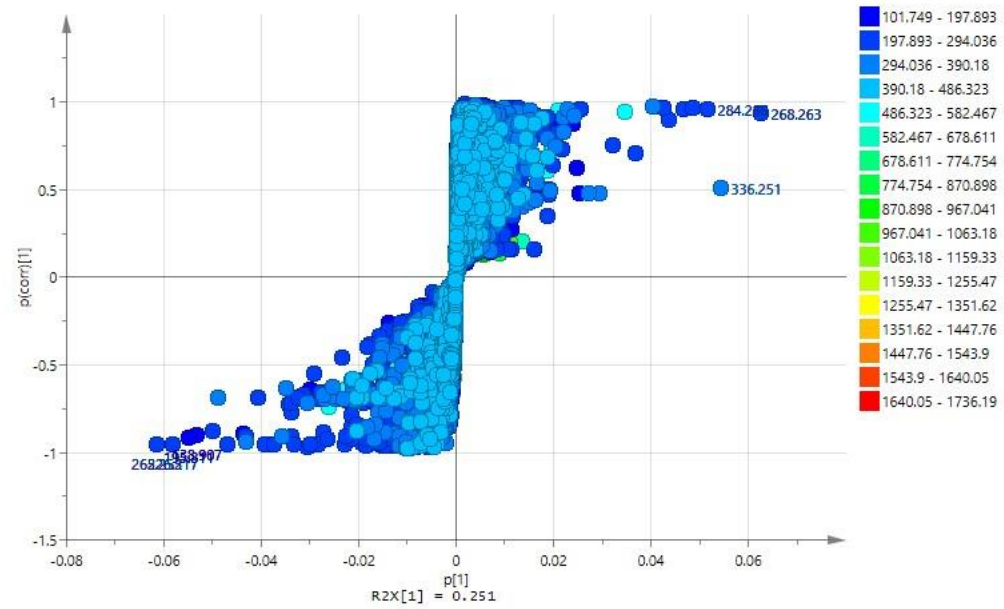


Figure 6.8: OPLS-DA S-plot exhibited metabolites on both Dynamis (upper right) and CD-CHO (lower left) groups (R2(Y) = 1; Q2(Y) = 0.96). (B) OPLS-DA S-plot exhibited metabolites on both CD-OPTICHO (lower left) and Dynamis (upper right) (R2(Y) = 0.98; Q2(Y) = 0.97). The unique metabolites found in all extract listed on **Table 6.5** and **Table 6.6**.

Table 6.5 Discriminating metabolites extracted from S-plot Figure 6.8 (A) in CD-CHO and Dynamis culture samples.

CD-CHO							
Primary ID	***m/z***	**P values P ≤ 0.05**	**RT (min)**	**Peak Area (b1-d5-xq)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LipidMaps /KEGG database**
N_3465	390.2499	0.9635	16.32	5.26E+07	391.2571	$C_{19}H_{37}NO_7$ [a]	No hits
N_3692	358.2599	0.9844	20.33	4.59E+05	335.2224	$C_{19}H_{37}NO_5$	2-hydroxylauroylcarnitine
P_37677	257.2667	0.9873	29.02	5.15E+07	256.2594	No prediction	No hits
Dynamis							
Primary ID	***m/z***	**P values P ≤ 0.05**	**RT (min)**	**Peak Area (D2D0)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LipidMApsLIPIDMAPS /KEGG database**
P_13891	256.8207	0.8000	1.13	2.13E+07	255.8134	No prediction	No hits
P_35137	278.2478	0.4923	23.36	5.68E+06	277.2405	$C_{18}H_{31}NO$	1-deoxy-(5E,9Z,12Z,15Z,d18:4) sphinganine
							[a] carnitine congener [b] sphingolipid congener

Table 6.6 Discriminating metabolites extracted from S-plot **Figure 6.8 (B)** in CD-OPTICHO and Dynamis culture samples.

CD-OPTICHO							
Primary ID	***m/z***	**P values P ≤ 0.05**	**RT (min)**	**Peak Area (optiB1D6)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LIPIDMAPS/KEGG database**
N_10844	195.8112	0.9183	1.19	3.36E+07	196.8185	No prediction	No hits
P_42075	138.9066	0.8983	1.11	2.59E+07	137.8993	No prediction	No hits
P_42077	226.2165	0.9527	22.87	3.62E+07	225.2092	$C_{14}H_{27}NO$	1-deoxy-(4E,6E,d14:2) sphinganine
P_35270	265.2525	0.9538	30.09	3.98E+07	264.2453	$C_{18}H_{32}O$	4-methyl-7,11-heptadecadienal
Dynamis							
Primary ID	***m/z***	**P values P ≤ 0.05**	**RT (min)**	**Peak Area (D1D11)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LIPIDMAPS/KEGG database**
P_35091	336.2507	0.5122	20.42	2.45E+02	335.2434	$C_{16}H_{29}N_7O$	No hits
P_35064	268.2634	0.9384	26.74	3.63E+07	267.2561	$C_{17}H_{33}NO$	1-deoxy-(4E,6E,d17:2) sphinganine
P_48306	284.2855	0.9592	28.60	2.98E+07	283.2782	No prediction	No hits

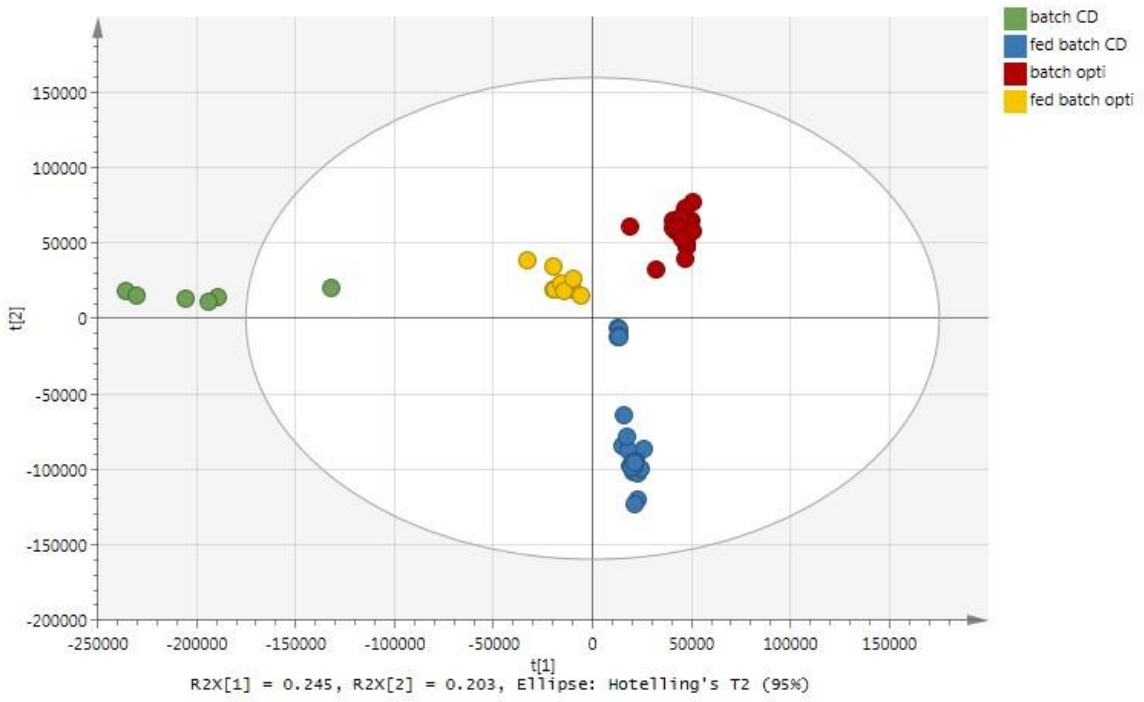
Metabolic profiling of CHO cells in different media has been done by Dietmair *et al*., (2012). Their study carried out in shake flask scale was a comparison using three commercially available culture media which are Hyclone (Thermo Fisher), CHOSFM (Invitrogen) and CDCHO (Invitrogen). They found that CHO cells cultivated in the three media displayed very different metabolic uptake rates. From all the analyses, cytidine triphosphate (CTP) was the only metabolite identified to be associated with different growth profile of the CHO cells. They did a confirmation study where they added CTP into CD-CHO cultures (lowest growth rate); however, adding CTP did not improve the growth rate. They highlighted that the example of CTP was one of the difficulties associated with the interpretation of metabolomics data. Subsequently Mohmad-Saberi *et al.,* (2013) also used a metabolomics approach to understand the relationship between types of medium used to culture growth behaviour and productivity. Their study (in T flasks working volume of 15 mL) involved two types of media (Ham's 12 and RPMI 1640 Cellgro USA). The results showed that each phase throughout the growth period had similar metabolites in different media, but with a different abundance. Their results were similar to the findings of the present study, where based on the abundance of metabolites, it was possible to separate the different types of growth media (Figure 6.4). Mohmad-Saberi *et al.,* (2013) also found out that ornithine and lysine (amino acids) were detected in the death phase of the cells cultured in RPMI medium and have been associated with apoptosis of cells.

Another potential use for metabolic profiling, other than looking to the metabolites that may occur during cell cultivations, is to examine whether or not medium taken from storage is in a good condition to be used to start a culture process. Zang *et al.*, (2011) investigated a medium lot used for a mammalian cell culture process that had resulted in low growth rate. They found out that an increased level of tryptophan oxidation products and a riboflavin degradant, lumichrome were in the malfunctioning medium lot which have the slowest growth rate and did not meet the expected viable cell density where it only gave a density of approximately 0.8×10^6 cells/mL.

6.3.3 Multivariate analysis for batch and fed batch bioreactor cultures using different media and feed nutrients.

In Chapter 5 the objective of the study was to gain a metabolic profile of the industrial GS-CHO cell lines by monitoring and preliminary screening of the relationship between the production of metabolites in different culture media (CD-CHO and CD-OPTICHO) and in different modes of cultivation (batch and fed batch). The unsupervised PCA scores plot (Figure 6.9 A) shows four clear clusters: CD-CHO medium batch mode, CD-OPTICHO medium batch mode, CD-CHO medium fed batch mode and lastly CD-OPTICHO medium fed batch mode. The loadings plot (Figure 6.9 B) displayed the distribution of metabolites for each cluster. The plot indicates that metabolites appearing in the left quadrant were produced in the culture of GS-CHO cells in CD-CHO medium in batch mode bioreactor. Then, the metabolic profile in the upper right quadrant represent metabolites from the culture of cells in CD-OPTI CHO medium batch mode while lower left quadrant are metabolites that produced in cultures of CD-CHO medium fed batch mode. Last but not least, the metabolites that appear in the culture of fed batch with CD-OPTICHO medium were in the middle of the upper quadrant.

(A)



(B)

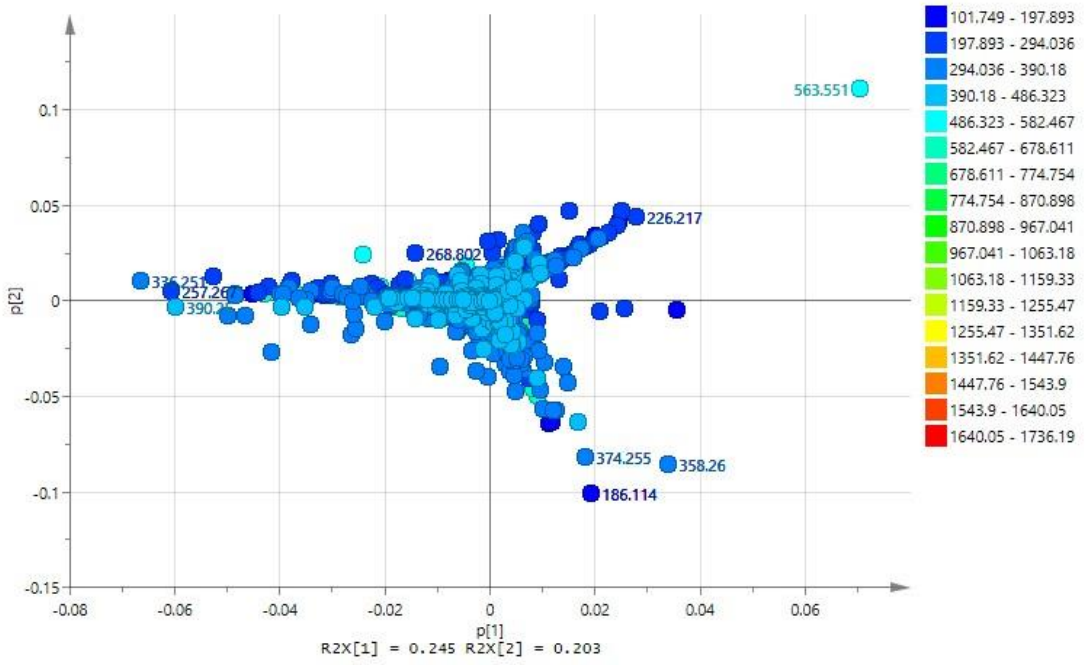


Figure 6.9: (A) PCA scores plot analysis of the GS-CHO cells showed distinctive separation between different medium (CD-CHO and CD-OPTICHO) with different mode of culture (batch and fed batch); (R2(Y) = (B) PCA loadings plot showed the distribution of the metabolites. The unique metabolites found in all extract listed on **Table 6.7**.

Table 6.7 Discriminating metabolites extracted from PCA loadings plot (Figure 6.9) in batch and fed batch with different medium culture samples.

Batch CD-CHO						
Primary ID	***m/z***	**RT (min)**	**Peak Area (b3-d5-xq)**	**Molecular Weight**	**Predicted MW**	**Putative hit from LIPIDMAPS /KEGG database**
N_3465	390.2499	16.32	6.60E+07	391.2571	$C_{19}H_{37}NO_7$ [a]	No hits
P_35091	336.2507	20.42	9.68E+07	335.2434	$C_{16}H_{29}N_7O$	No hits
P_37677	257.2667	29.02	7.54E+07	256.2594	No prediction	No hits
Fed batch CD-CHO						
Primary ID	***m/z***	**RT (min)**	**Peak Area (fb1d3x)**	**Molecular Weight**	**Predicted MW**	**Putative hit from LIPIDMAPS /KEGG database**
N_10836	186.1139	6.93	1.22E+08	187.1211	$C_9H_{17}NO_3$	8-amino-7-oxononanoate
N_10852	374.2551	16.40	1.12E+08	375.2624	$C_{19}H_{37}NO_6$ [a]	No hits
N_10837	358.2599	22.21	1.43E+08	359.2671	$C_{19}H_{37}NO_5$	2-hydroxylauroylcarnitine
Batch CD-OPTICHO						
Primary ID	***m/z***	**RT (min)**	**Peak Area (optiB1D4)**	**Molecular Weight**	**Predicted MW**	**Putative hit from LIPIDMAPS /KEGG database**
P_42077	226.2165	22.87	4.10E+07	225.2092	$C_{14}H_{27}NO$	1-deoxy-(4E,6E,d14:2) sphinganine
P_42074	563.5506	30.46	2.56E+08	562.5433	$C_{36}H_{70}N_2O_2$ [b]	No hits
Fed batch CD-OPTICHO						
Primary ID	***m/z***	**RT (min)**	**Peak Area (FBO1D1)**	**Molecular Weight**	**Predicted MW**	**Putative hit from LIPIDMAPS /KEGG database**
N_15663	268.8020	1.13	3.69E+07	269.8092	No prediction	No hits

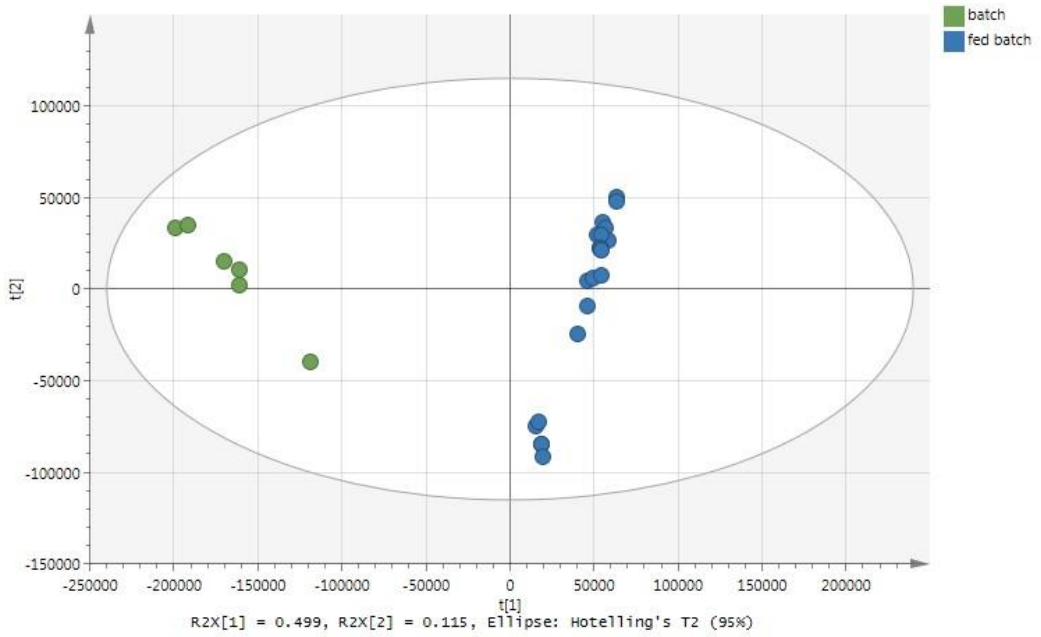
[a] carnitine congener
[b] sphingolipid congener

The unsupervised PCA analysis also was done within the same culture medium with different modes of cultivation. Figure 6.10 (A) shows distinctive separation between batch and fed batch mode of culture in CD-CHO medium. The loadings plot (Figure 6.10 B) shows the distributed metabolites produce in the two different culture modes where the right quadrant are metabolites for the fed batch culture mode, while the left quadrant represent the metabolites from batch culture mode. Similarly, Figure 6.11 A shows a good separation between batch and fed batch culture mode in CD-OPTICHO medium. The loadings plot (Figure 6.11 B) shows the distribution of metabolites for each mode of culture in the different part of the quadrant.

Sellick *et al.,* (2011) used metabolite profiling to define the balance of intracellular and extracellular metabolites during the production process of a CHO cell line expressing a recombinant IgG4 antibody. They managed to identify nutrient limitations, which acted as bottlenecks for antibody production, and subsequently developed a simple feeding regime to relieve these metabolic bottlenecks. In addition, the metabolite profiling-based strategy was used to design a targeted, low cost nutrient feed that increased cell biomass by 35% and doubled the antibody titre. Furthermore, Sellick *et al*., (2015) used both intracellular and extracellular metabolite profiling, to examine the metabolic consequences by the addition of two commercial feeds (EFA and EFB) on cellular performance. They mentioned that the cell system did continued producing protein even in an unfed environment but the introduction of either EFA or EFB to the culture environment does increase the rate of accumulation of protein during the stationary phase of culture. The amount of amino acids were increased by multiple fold; where serine and threonine were among the two which are markedly increased by addition of the nutrient feeds to the culture. Serine and threonine were

used as energy feeds for the citric acid cycle throughout the later stages of fed batch cultures thus provide a prolong survival of cells under the fed condition. In addition, intermediates for citric acid cycle (malate, fumarate, citrate and succinate) also were found to be increase with the addition of nutrient feed to the culture. That condition extended the cell culture growth and survival.

(A)



(B)

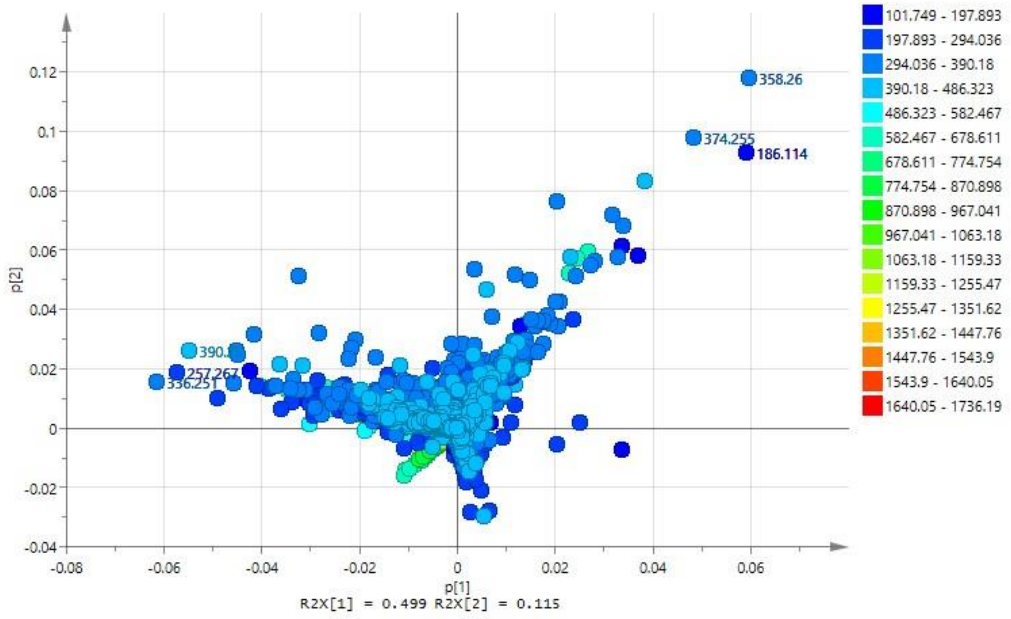
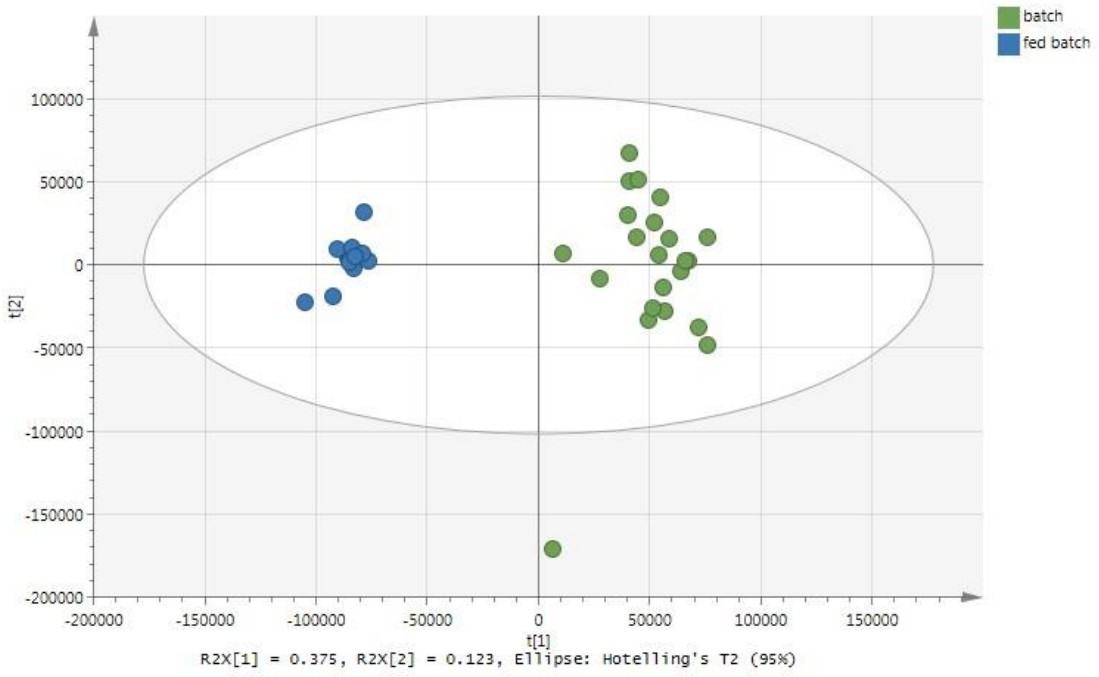


Figure 6.10: PCA analysis GS-CHO cells cultured in CD-CHO medium in batch and fed batch mode. (A) PCA scores plot analysis of the GS-CHO cells showed distinctive separation between different mode of culture; (R2(Y) = 0.61; Q2(Y) = 0.54) (B) PCA loadings plot showed the distribution of the metabolites. The unique metabolites found in all extract listed on **Table 6.8**.

(A)



(B)

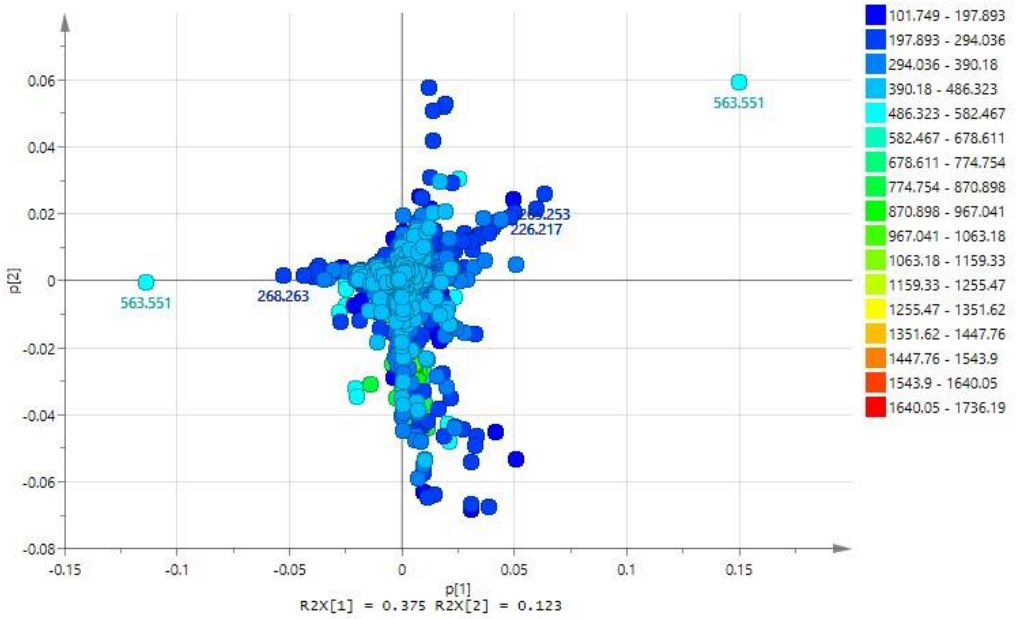
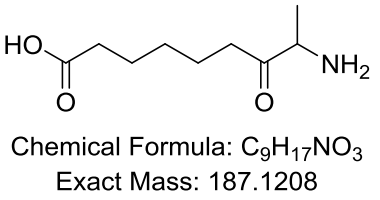


Figure 6.11: PCA analysis of GS-CHO cells cultured in CD-OPTICHO medium in batch and fed batch mode. (A) PCA scores plot of the GS-CHO cells showed distinctive separation between different mode of culture; (R2(Y) = 0.65; Q2(Y) = 0.46; (B) PCA loadings plot showed the distribution of the metabolites. The unique metabolites found in all extract listed on **Table 6.8**.

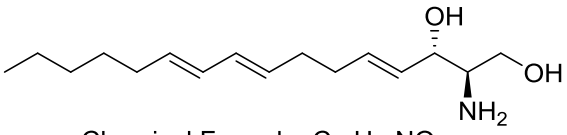
Table 6.8 Discriminating metabolites extracted from S-plot Figure 6.11 in batch and fed batch with CD-OPTICHO media culture samples.

Batch CD-OPTICHO						
Primary ID	***m/z***	**RT (min)**	**Peak Area (optiB1D4)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LIPIDMAPS/KEGG database**
P_42077	226.2165	22.87	4.10E+07	225.2092	$C_{14}H_{27}NO$	1-deoxy-(4E,6E,d14:2) sphinganine
P_35270	265.2525	30.09	4.82E+07	264.2453	$C_{18}H_{32}O$	4-methyl-7,11-heptadecadienal
P_42074	563.5506	30.46	2.56E+08	562.5433	$C_{36}H_{70}N_2O_2$ [b,*]	No hits
Fed batch CD-OPTICHO						
Primary ID	**m/z**	**RT (min)**	**Peak Area (FBO1D1)**	**Molecular Weight**	**Predicted Molecular Formula**	**Putative hit from LIPIDMAPS/KEGG database**
P_35064	268.2634	26.74	4.47E+07	267.2561	$C_{17}H_{33}NO$	1-deoxy-(4E,6E,d17:2) sphinganine
P_35072	563.5514	28.65	2.64E+08	562.5442	$C_{36}H_{70}N_2O_2$ [b,*]	No hits
						[a] carnitine congener [b] sphingolipid congener



Chemical Formula: $C_9H_{17}NO_3$
Exact Mass: 187.1208

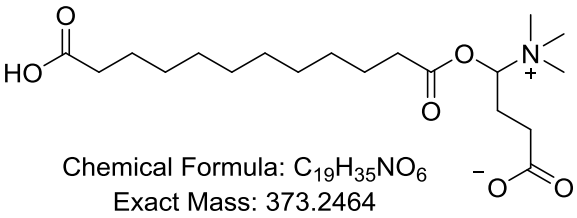
8-amino-7-oxononanoate



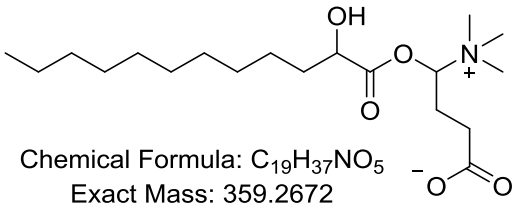
Chemical Formula: $C_{16}H_{29}NO_2$
Exact Mass: 267.2198

(4E,8E,10E-d16:3) sphingosine

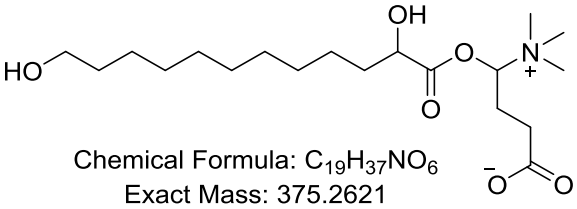
carnitine congeners



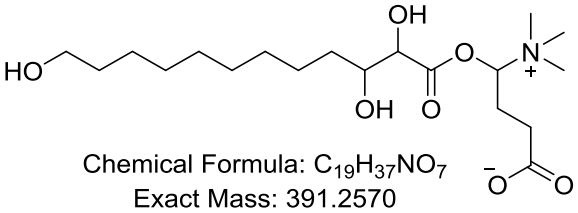
Chemical Formula: $C_{19}H_{35}NO_6$
Exact Mass: 373.2464



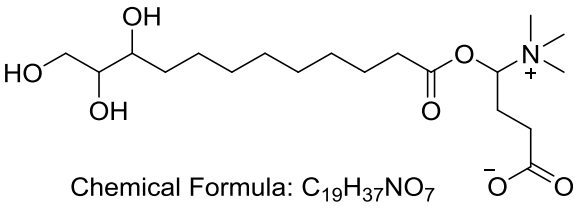
Chemical Formula: $C_{19}H_{37}NO_5$
Exact Mass: 359.2672



Chemical Formula: $C_{19}H_{37}NO_6$
Exact Mass: 375.2621



Chemical Formula: $C_{19}H_{37}NO_7$
Exact Mass: 391.2570



Chemical Formula: $C_{19}H_{37}NO_7$
Exact Mass: 391.2570

Figure 6.12: Putative structures of compounds found in GS-CHO cells cultured in bioreactor with CD-CHO medium.

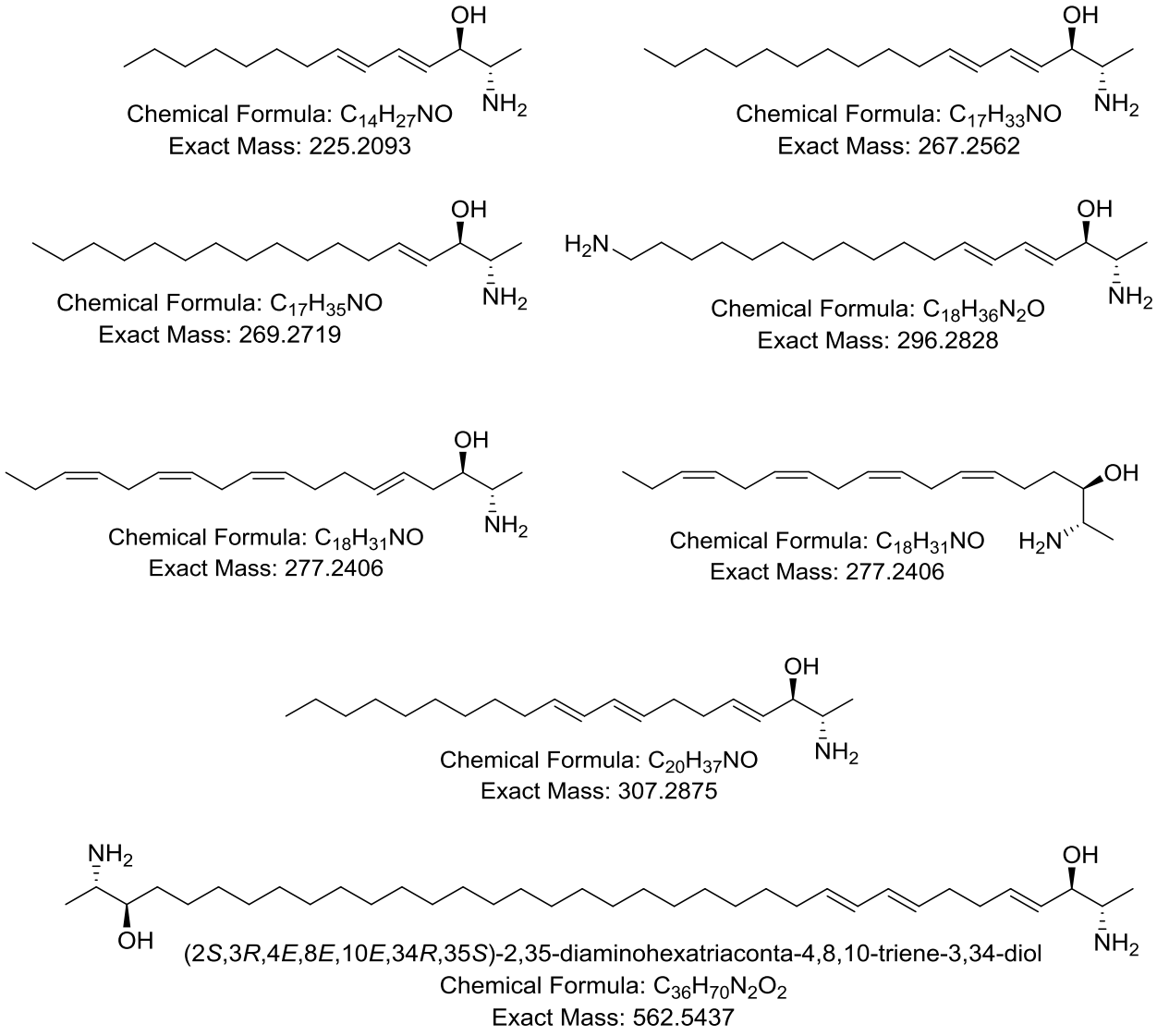


Figure 6.13: Putative structures of sphingolipid congeners found in GS-CHO cells cultured in shake flask and bioreactor with CD-OptiCHO and Dynamis media.

Table 6.9 Summary of expressed biosynthetic pathway as obtained from the discriminating metabolites by metabolomic profiling.

Fermentation Condition	Biosynthetic Pathway Expression		
	Carnitine	**Sphingolipid**	**Biotin**
Shake Flask	decrease	increase	decrease
Bioreactor	increase	decrease	increase
CD-CHO	increase	decrease	increase
CD-OptiCHO	decrease	increase	decrease
Dynamis	decrease	increase	decrease
CD-CHO Batch	increase	decrease	decrease
CD-CHO Fed-Batch	increase	decrease	increase
CD-OptiCHO Batch	decrease	increase	decrease
CD-OptiCHO Fed-Batch	decrease	increase	decrease

Table 6.9 shows a summary of expressed biosynthetic pathway as indicated by the discriminating metabolites by metabolic profiling. It can be seen that three biosynthetic pathways (carnitine, sphingolipid and biotin) were either upregulated (expressed) or downregulated by the different culture conditions and media. Carnitine biosynthesis is crucial for energy metabolism, where it allows fatty acids to enter the mitochondria after being broken down via β-oxidation. Next is sphingolipid biosynthesis is an essential component of eukaryotic cell membranes (Jafarulla *et al.*, 2016). Sphingolipid is known to modulate a variety of cellular functions including the integral membrane proteins such as G protein-coupled receptors (GPCRs). The third

discriminating metabolites that had been detected were involved in biotin synthesis. Biotin is also known as vitamin H and B7 which is an important enzyme cofactor that is necessary for needed steps of central metabolism involved in fatty acid synthesis and amino acid degradation.

From Table 6.9 it can be observed that there are regulation patterns for the three biosynthetic pathways in different types of culture (shake flask and bioreactor), different medium (CD-CHO, CD-OPTICHO and Dynamis) and different mode of culture (batch and fed batch). Sphingolipid biosynthesis seems to increase in shake flask culture while it is down regulated in the bioreactor culture. Carnitine and biotin biosynthesis have a similar pattern where both were down regulated in the shake flask culture and increased or upregulated in the bioreactor culture. While for culture of GS-CHO in different media, CD-OPTICHO and Dynamis culture have similar pattern for the discriminating metabolites involving down regulation of both carnitine and biotin biosynthesis while sphingolipid biosynthesis was upregulated. A different trend was observed for the cultures from CDCHO media where an increased biosynthesis of carnitine and biotin while sphingolipid biosynthesis was decreased. In the different culture mode (batch and fed batch), a rising trend was observed for carnitine production in both batch and fed batch culture in CD-CHO media while it was decreased in the CD-OPTICHO media culture. An opposite reaction was exhibited for sphingolipid biosynthesis where it decrease in CD-CHO media batch and fed batch while it increased in CD-OPTICHO media culture. On the other hand, biotin production seems to be different in both culture mode, it was increased in a fed batch mode and decreased in a batch mode. That aside, GS-CHO cultured in CD-OPTICHO media showed similar trend for the three biosynthetic pathways in both batch and fed

batch mode of culture. Carnitine and biotin biosynthesis declined while sphingolipid biosynthesis seems to be enhanced. Further investigation can be made in the future on what this three discriminating metabolites could affect the culture of GS-CHO and more importantly the production of IgG.

6.4 Conclusion

A metabolomics approach was shown to be applicable to the identification of chemical changes occurring in cell culture associated with low cell growth and low product yield in a GS-CHO cell lines manufacturing process. Results of the metabolomics analysis provided some understanding of the different scales of culture, different modes of culture and different media that will show performance differences, with regard to the industrial GS-CHO cell lines under study. Specifically, there are clear differences in the metabolic profile of those above mentioned culture conditions. Current state-of-the-art commercial LC-MS systems were used to profile the biological metabolism of CHO.

In the first part of this study, LC-MS-based untargeted metabolomics has shown to be a valuable tool for characterizing the different metabolic profile in different scale of culture (shake flask and bioreactor). The multivariate analysis in this study clearly helped explain the culture differences and understand the system as a whole. Collectively, the metabolic profile of the different scale of the cultures (shake flask and bioreactor) were distinct.

In the second part of the chapter, metabolomics tools was successfully applied in three different commercially available chemically defined media in batch bioreactor culture to time-profile intracellular metabolites in the culture environment. The analysis has shown that different growth media have different effects on growth behaviour. Different types of media also appeared to affect IgG production.

Finally, metabolomics analysis of different culture modes of GS-CHO cell culture (batch and fed batch) also showed distinct metabolic profiles for each. The results revealed that different metabolites were appearing in those different culture conditions and they were associated with different physiological behaviour of each GS-CHO cell culture. These metabolites can be identified given more time for analysing.

The findings from such an analysis pave the way for a better industrial GS-CHO culture environment in two aspects. The first involves looking into the best available chemically defined medium for GS-CHO cell lines and what are the metabolic profiles for the in different media before use. The second aspect involves knowing the metabolic profile which evolves in various culture modes where medium limitations which might lead to underperformance can be remedied. Such efforts will likely result in improved CHO cell growth and product titre. In addition, results attained from the analysis could possibly contribute towards the development of a mass-balance model. This will help in the identification of critical reaction pathways which could be modified for optimal cellular metabolism of GS-CHO cell cultures.

Furthermore, using the results revealed in this study, several bioprocessing strategies including medium improvement, feeding strategy and downstream processing can be potentially implemented to achieve efficient CHO culture system. Nevertheless, more

detailed studies are warranted to confirm and complement the existing information. The results in this work show how important process related information can be obtained with generally applicable univariate and multivariate process analysis methods. Especially in cell culture process development, which is characterized by lengthy run times, a large number of influential and mutually interacting factors, as well as high-cost raw materials and process analytics, multivariate data analysis represents an attractive and versatile tools for process development.

Chapter 7

Conclusions and Recommendations

This chapter aims are to review and summarise the key findings of each chapter and provide future work recommendations. Each chapter will be individually assessed for industrially relevant findings concerning the GS-CHO cell lines supplied by Lonza. Future work recommendations will then be made based upon the overall findings.

This project involved assessment of the performance of two industrial CHO cell lines, under a range of culture conditions. Conditions investigated included the effects of passage number of a single cell line, effects of culture media/ feed types, reactor types (shake flask and bioreactor) and mode of cultures (batch and fed batch). A metabolomics approach was investigated at the end of this project to observe the metabolic profiles displayed by the culture under each set of conditions described above.

7.1 Characterisation of the physiology of industrial CHO cell lines in shake flask batch cultures.

The results presented in Chapter 3 showed that the physiology of a high IgG producer and low IgG producer cell lines were different. Although the cell density of the low IgG producer cell line was higher, it was not significantly different to the cell density of higher IgG producer as tested by the T-test (section 3.3.1). The extracellular concentration of the five key nutrients which are the glucose, lactate, glutamine, ammonia and glutamate are different for the growth and stationary phase. In addition, different passage number of the same clone of cell cultured in different culture media

were being tested. The lower passage number (4) cultured in CD-CHO medium had the highest cell density and IgG production compared to the higher passage number (17) and also compared to CD-OPTICHO medium culture. The study shows clearly that medium type and passage number both profoundly influenced the physiology of the industrial GS-CHO cell lines. As a result, GS-CHO 42 passage number 4 were chosen as the starting point in the subsequent studies.

7.2 Characterisation of physiology of industrial CHO cell lines in bioreactor batch cultures.

Chapter 4 centred around assessing the physiology of GS-CHO 42 passage number 4 in bioreactor batch cultures using three commercially available culture media (CD-CHO, CD-OPTICHO and Dynamis). From the results, cell density and IgG production of GS-CHO cells were best in cells cultured in CD-CHO medium. CD-OPTICHO media and Dynamis media are also media that were developed for GS-CHO cell lines but CD-CHO outperformed them both here. Although GS-CHO cells can also grow in both of the media, lengthier time is needed for the adaptation process to ensure high cell density and high productivity will be achieved. In addition, a comparison of shake flask and bioreactor operating strategies are discussed in Chapter 4. This is to recognise whether shake flask culture are comparable to the bioreactor culture as shake flask system in an uncontrollable environment compare to a bioreactor system. It can be seen that shake flask cultures have higher cell density, lower IgG production while bioreactor culture have lower density of cells with higher IgG concentration. The

differences of cell density and IgG production from shake flask culture and bioreactor culture were significant as tested earlier (section 4.3). The results are important because the author's intention is to have a small scale system (shake flask) that can mimic the upscale system (bioreactor) using the same cell line. However, the study shows significant difference between the two scales of cultures concluding that shake flask study could not be used as a simple optimisation step for predicting performance in the bioreactor.

7.3 Characterisation of the physiology of industrial CHO cell lines in bioreactor fed batch cultures.

In Chapter 5, GS-CHO 42 passage 4 cells was tested in a fed batch system using two media (CD-CHO and CD-OPTICHO) with two nutrient feed supplements (EFA and EFB) that were also supplied by the industrial partner of the project. The cells show different metabolic and physiological responses for the different medium-feed combinations that were applied. Results showed that the combination of CD-CHO medium with EFB gave a higher cell density and IgG production compared to CD-OPTICHO medium + EFA. However, given more time, the GS-CHO cells could be adapted to CD-OPTICHO media with EFA and better performance might result. The conclusion of this chapter is that the systematic comparison of two media and supplements needs more extensive knowledge of the process of cell line adaptation to novel media/feed regimes than was possible in this study.

7.4 Metabolomics analysis of industrial CHO cell lines in different culture environment.

Chapter 6 of the thesis used metabolomics analysis to look at the metabolic profile of each of the culture from Chapter 3, 4, and 5. Multivariate analysis (PCA and OPLS-DA) showed that the metabolic profiles of the different scales of cultures (shake flask and bioreactor) were distinct. That is in agreement with the differences in the physiology of these cultures seen in Chapter 4. This chapter also reveals that growth medium/feed and culture mode (batch and fed batch) have distinct effects on the metabolic profile of the GS-CHO cells. The author is confident that if more time of analysis was possible, a significant number of metabolites of interest can be identified. Thus more insight into the relationship between culture conditions and metabolism could be established.

7.5 Recommendations

The work carried out in this study shows that shake flask cultures are significantly different to the upscale culture system in the bioreactor. The capacity of the shake flask based systems to mimic the performance of conventional lab-scale bioreactor (5L) may not gave fundamental insight into the bioreactor performance. However, as mentioned in the earlier part of the thesis (section 3.1) where study at a larger scale will have a very high cost, the essential of having a small scale study is very much needed. It is recommended that in future study, small scale bioreactor system can be explored and applied for medium and feed optimisation study. Some examples of small scale bioreactor that have been used by other researchers are the small scale bioreactor (Bareither *et al*., 2013), Microwell based system and miniature bioreactor (Sani, 2015) and Scale-up Ambr 250 disposable bioreactors (Xu *et al*., 2017). Each of these system investigated have the potential to perform well in early process development and optimisation studies. (Bareither *et al*., 2013) provided that the small scale disposable bioreactor data generated in their study have equivalent process performance for industrial biologics processes for therapeutic protein and monoclonal antibody production using CHO cell culture, growth, cell viability, product titre, and product quality. In addition Sani (2015) also confirmed that Microwell based Systems and Miniature Bioreactors can be used as an alternative to mimic the operation of conventional benchtop bioreactors. Another example is the Ambr 250 disposable bioreactors which offers several advantages including highly automated control, high throughput capacity and short turnaround time (Xu *et al*., 2017). Their study showed that cell growth, productivity, metabolite profiles, and product qualities of material

generated using the Ambr 250 were comparable to those from 5-L bioreactors. Furthermore, these systems could offer optimisation studies for media and feed development. The research presented here, (using shake flask) does offer a foundation from which future developments can be made. Due to both the time limitations and some of the unexpected results, there are a number of aspects which would benefit from future improvements and developments. Therefore, future prospect of the project would be incorporating f small scale system to study the physiology and metabolome of the industrial GS-CHO cell lines to ensure a more complete understanding of the links between physiology and metabolism across scales. This would generate more confidence and speed the scale-up process. .

On the other hand, for batch bioreactor study, (Chapter 4), the GS-CHO cell lines can be adapted to Dynamis medium. The adaptation phase may take up to 6 months of work to produce a reliable result in term of cell density and product quality. Therefore it is suggested that in any future study of the industrial GS-CHO cell line will focus more on the adaptation of cell to the newly developed chemically defined medium because the medium does offer advantages for the CHO cell culture. As mentioned in the manufacturer's website Dynamis medium is specifically designed to offer the highest batch and fed-batch culture performance and yield with recombinant CHO cells in a chemically defined environment (www.thermofisher.com).

Whereas fed batch study can be focus on using CD-OPTICHO medium because the manufacturer claimed that it is specifically designed to offer high performance and yield with recombinant CHO cells in a chemically defined fed-batch environment with consistent cell growth and titres, and offering up to 40% better titre and cell growth over some other medium (www.thermofisher.com). Feeding regimes of EFA

and EFB also should be optimised by increasing the number of fed-batch runs and varying the feed regimes because this project only applied the feeding regimes from Barrett *et al*., (2012).

The metabolic profiles of GS-CHO cell cultures under a range of process conditions has been revealed in this study. The next logical step would be to obtain a global profile of intracellular metabolite changes within the different culture conditions. Methods specific to GS-CHO cell lines would have to be developed with regards to quenching of metabolism and extraction of intracellular metabolites with good and reproducible recoveries. The data would provide some answers to the earlier observations in Dynamis media and fed batch culture system of why the cells did not meet the standard of a good culture conditions with higher cell density and IgG productivity. Such information will provide a better picture of the chain of events leading to a cellular apoptosis so that the point of intervention can be more precisely located.

In addition to highlighting the potential of metabolomics, this study also demonstrates the difficulties associated with interpretation of metabolomics data. This is because the metabolomics datasets are very large and described by multiple variables. Therefore, bioinformatics solutions with statistical tools are essential to reduce the complexity and highlight interesting features. Metabolic profile of an industrial GS-CHO cell lines will be useful to reflect the sequential steps in a biochemical pathway, including translocation out of and into the cell where applicable.

References

Aggarwal, S., 2014. What's fueling the biotech engine—2012 to 2013. Nature Biotechnology 32, 32.

Aghamohseni, H., Ohadi, K., Spearman, M., Krahn, N., Moo-Young, M., Scharer, J. M., Butler, M., Budman, H. M., 2014. Effects of nutrient levels and average culture pH on the glycosylation pattern of camelid-humanized monoclonal antibody. J Biotechnol 186, 98-109.

Ahn, W. S., Antoniewicz, M. R., 2011. Metabolic flux analysis of CHO cells at growth and non-growth phases using isotopic tracers and mass spectrometry. Metabolic Engineering 13, 598-609.

Ahuja, S., Jain, S., Ram, K., 2015. Application of multivariate analysis and mass transfer principles for refinement of a 3-L bioreactor scale-down model—when shake flasks mimic 15,000-L bioreactors better. Biotechnology progress 31, 1370-1380.

Altamirano, C., Paredes, C., Cairo, J., Godia, F., 2000. Improvement of CHO cell culture medium formulation: simultaneous substitution of glucose and glutamine. Biotechnology progress 16, 69-75.

Altamirano, C., Paredes, C., Illanes, A., Cairo, J. J., Godia, F., 2004. Strategies for fed-batch cultivation of t-PA producing CHO cells: substitution of glucose and glutamine and rational design of culture medium. J Biotechnol 110, 171-179.

Badsha, M. B., Kurata, H., Onitsuka, M., Oga, T., Omasa, T., 2016. Metabolic analysis of antibody producing Chinese hamster ovary cell culture under different stresses conditions. Journal of bioscience and bioengineering 122, 117-124.

Baik, J. Y., Dahodwala, H., Oduah, E., Talman, L., Gemmill, T. R., Gasimli, L., Datta, P., Yang, B., Li, G., Zhang, F., 2015. Optimization of bioprocess conditions improves production of a CHO cell-derived, bioengineered heparin. Biotechnology journal 10, 1067-1081.

Bareither, R., Bargh, N., Oakeshott, R., Watts, K., Pollard, D., 2013. Automated disposable small scale reactor for high throughput bioprocess development: a proof of concept study. Biotechnology and bioengineering 110, 3126-3138.

Barrett, S. L., Boniface, R., Dhulipala, P., Slade, P., Tennico, Y., Stramaglia, M., Lio, P., Gorfien, S., 2012. Attaining Next-Level Titers in CHO Fed-Batch Cultures. BioProcess International 10, 56-62.

Becerra, S., Berrios, J., Osses, N., Altamirano, C., 2012. Exploring the effect of mild hypothermia on CHO cell productivity. Biochemical Engineering Journal 60, 1-8.

Beckmann, T. F., Kramer, O., Klausing, S., Heinrich, C., Thute, T., Buntemeyer, H., Hoffrogge, R., Noll, T., 2012. Effects of high passage cultivation on CHO cells: a global analysis. Appl Microbiol Biotechnol 94, 659-671.

Bradley, S. A., Ouyang, A., Purdie, J., Smitka, T. A., Wang, T., Kaerner, A., 2010. Fermentanomics: monitoring mammalian cell cultures with NMR spectroscopy. J Am Chem Soc 132, 9531-9533.

Bro, R., Smilde, A. K., 2014. Principal component analysis. Analytical Methods 6, 2812-2831.

Campbell, C. E., Worton, R. G., 1979. Evidence obtained by induced mutation frequency analysis for functional hemizygosity at theemt locus in CHO cells. Somatic Cell and Molecular Genetics 5, 51-65.

Carinhas, N., Duarte, T. M., Barreiro, L. C., Carrondo, M. J., Alves, P. M., Teixeira, A. P., 2013. Metabolic signatures of GS-CHO cell clones associated with butyrate treatment and culture phase transition. Biotechnol Bioeng 110, 3244-3257.

Carrillo-Cocom, L., Genel-Rey, T., Araiz-Hernandez, D., Lopez-Pacheco, F., Lopez-Meza, J., Rocha-Pizana, M., Ramirez-Medrano, A., Alvarez, M., 2015. Amino acid consumption in naive and recombinant CHO cell cultures: producers of a monoclonal antibody. Cytotechnology 67, 809-820.

Castilho, L., 2016. Biopharmaceutical Products: An Introduction. Current Developments in Biotechnology and Bioengineering: Human and Animal Health Applications, 1.

Carvalho, L. S., da Silva, O. B., de Almeida, G. C., de Oliveira, J. D., Parachin, N. S., Carmo, T. S., 2017. Production Processes for Monoclonal Antibodies. Fermentation Processes. InTech.

Çelik, E., Çalık, P., 2012. Production of recombinant proteins by yeast cells. Biotechnology advances 30, 1108-1118.

Chabanon, G., da Costa, L. A., Farges, B., Harscoat, C., Chenu, S., Goergen, J. L., Marc, A., Marc, I., Chevalot, I., 2008. Influence of the rapeseed protein hydrolysis process on CHO cell growth. Bioresource technology 99, 7143-7151.

Chee Furng Wong, D., Tin Kam Wong, K., Tang Goh, L., Kiat Heng, C., Gek Sim Yap, M., 2005. Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures. Biotechnol Bioeng 89, 164-177.

Chong, W. P., Goh, L. T., Reddy, S. G., Yusufi, F. N., Lee, D. Y., Wong, N. S., Heng, C. K., Yap, M. G., Ho, Y. S., 2009. Metabolomics profiling of extracellular metabolites in recombinant Chinese Hamster Ovary fed-batch culture. Rapid Commun Mass Spectrom 23, 3763-3771.

Chong, W. P. K., Yusufi, F. N. K., Lee, D.-Y., Reddy, S. G., Wong, N. S. C., Heng, C. K., Yap, M. G. S., Ho, Y. S., 2011. Metabolomics-based identification of apoptosis-inducing metabolites in recombinant fed-batch CHO culture media. Journal of biotechnology 151, 218-224.

Croset, A., Delafosse, L., Gaudry, J.-P., Arod, C., Glez, L., Losberger, C., Begue, D., Krstanovic, A., Robert, F., Vilbois, F., Chevalet, L., Antonsson, B., 2012. Differences in the glycosylation of recombinant proteins expressed in HEK and CHO cells. Journal of biotechnology 161, 336-348.

Datta, P., Linhardt, R. J., Sharfstein, S. T., 2013. An 'omics approach towards CHO cell engineering. Biotechnol Bioeng 110, 1255-1271.

Dietmair, S., Hodson, M. P., Quek, L. E., Timmins, N. E., Chrysanthopoulos, P., Jacob, S. S., Gray, P., Nielsen, L. K., 2012. Metabolite profiling of CHO cells with different growth characteristics. Biotechnol Bioeng 109, 1404-1414.

Dietmair, S., Timmins, N. E., Gray, P. P., Nielsen, L. K., Kromer, J. O., 2010. Towards quantitative metabolomics of mammalian cells: development of a metabolite extraction protocol. Anal Biochem 404, 155-164.

Dorai, H., 2013. Proteomic Analysis of Bioreactor Cultures of an Antibody Expressing CHOGS Cell Line that Promotes High Productivity. Journal of Proteomics & Bioinformatics 06, 99-108.

Duarte, T. M., Carinhas, N., Barreiro, L. C., Carrondo, M. J., Alves, P. M., Teixeira, A. P., 2014. Metabolic responses of CHO cells to limitation of key amino acids. Biotechnol Bioeng 111, 2095-2106.

Erac, Y., Selli, C., Filik, P., Tosun, M., 2014. Effects of passage number on proliferation and store-operated calcium entry in A7r5 vascular smooth muscle cells. Journal of pharmacological and toxicological methods 70, 1-5.

Fernandez Martell, A., 2015. Evaluation and characterisation of phenotypic heterogeneity in Chinese Hamster Ovary cell populations during long-term culture. University of Sheffield.

Florin, L., Lipske, C., Becker, E., Kaufmann, H., 2011. Supplementation of serum free media with HT is not sufficient to restore growth properties of DHFR-/- cells in fed-batch processes - Implications for designing novel CHO-based expression platforms. Journal of biotechnology 152, 189-193

Floris, P., McGillicuddy, N., Albrecht, S., Morrissey, B., Kaisermayer, C., Lindeberg, A., Bones, J., 2017. Untargeted LC-MS/MS Profiling of Cell Culture Media Formulations for Evaluation of High Temperature Short Time Treatment Effects. Analytical chemistry 89, 9953-9960.

Forabosco, F., Löhmus, M., Rydhmer, L., Sundström, L. F., 2013. Genetically modified farm animals and fish in agriculture: A review. Livestock Science 153, 1-9.

Freitas, V. J. F., Serova, I. a., Moura, R. R., Andreeva, L. E., Melo, L. M., Teixeira, D. I. a., Pereira, a. F., Lopes-Jr, E. S., Dias, L. P. B., Nunes-Pinheiro, D. C. S., Sousa, F. C., Alcântara-Neto, a. S., Albuquerque, E. S., Melo, C. H. S., Rodrigues, V. H. V., Batista, R. I. T. P., Dvoryanchikov, G. a., Serov, O. L., 2012. The establishment of two transgenic goat lines for mammary gland hG-CSF expression. Small Ruminant Research 105, 105-113.

Gika, H. G., Wilson, I. D., Theodoridis, G. A., 2014. LC–MS-based holistic metabolic profiling. Problems, limitations, advantages, and future perspectives. Journal of Chromatography B 966, 1-6.

González-Leal, I. J., Carrillo-Cocom, L. M., Ramírez-Medrano, a., López-Pacheco, F., Bulnes-Abundis, D., Webb-Vargas, Y., Alvarez, M. M., 2011. Use of a plackett-burman statistical design to determine the effect of selected amino acids on monoclonal antibody production in CHO cells. Biotechnology Progress 27, 1709-1717.

Ha, T. K., Lee, G. M., 2014. Effect of glutamine substitution by TCA cycle intermediates on the production and sialylation of Fc-fusion protein in Chinese hamster ovary cell culture. Journal of Biotechnology 180, 23-29.

Han, Y. K., Ha, T. K., Lee, S. J., Lee, J. S., Lee, G. M., 2011. Autophagy and apoptosis of recombinant Chinese hamster ovary cells during fed-batch culture: effect of nutrient supplementation. Biotechnology and bioengineering 108, 2182-2192.

Hakemeyer, C., Strauss, U., Werz, S., Jose, G. E., Folque, F., Menezes, J. C., 2012. At-line NIR spectroscopy as effective PAT monitoring technique in Mab cultivations during process development and manufacturing. Talanta 90, 12-21.

Hazeltine, L. B., Knueven, K. M., Zhang, Y., Lian, Z., Olson, D. J., Ouyang, A., 2015. Chemically defined media modifications to lower tryptophan oxidation of biopharmaceuticals. Biotechnology progress.

Hefferon, K. L., 2012. Plant virus expression vectors set the stage as production platforms for biopharmaceutical proteins. Virology 433, 1-6.

Hernández Bort, J. a., Shanmukam, V., Pabst, M., Windwarder, M., Neumann, L., Alchalabi, A., Krebiehl, G., Koellensperger, G., Hann, S., Sonntag, D., Altmann, F., Heel, C., Borth, N., 2014. Reduced quenching and extraction time for mammalian cells using filtration and syringe extraction. Journal of Biotechnology 182-183, 97-103.

Heinrich, C., Wolf, T., Kropp, C., Northoff, S., Noll, T., 2011. Growth characterization of CHO DP-12 cell lines with different high passage histories. BMC Proceedings 5, P29.

Henry, O., Durocher, Y., 2011. Enhanced glycoprotein production in HEK-293 cells expressing pyruvate carboxylase. Metabolic engineering 13, 499-507.

Hong, J. K., Cho, S. M., Yoon, S. K., 2010. Substitution of glutamine by glutamate enhances production and galactosylation of recombinant IgG in Chinese hamster ovary cells. Applied Microbiology and Biotechnology 88, 869-876.

Hossler, P., Wang, M., McDermott, S., Racicot, C., Chemfe, K., Zhang, Y., Chumsae, C., Manuilov, A., 2015. Cell culture media supplementation of bioflavonoids for the targeted reduction of acidic species charge variants on recombinant therapeutic proteins. Biotechnology progress 31, 1039-1052.

Hounoum, B. M., Blasco, H., Emond, P., Mavel, S., 2015. Liquid chromatography-high resolution mass spectrometry-based cell metabolomics: experimental design, recommendations and applications. TrAC Trends in Analytical Chemistry.

Huang, C., Jr., Lowe, A. J., Batt, C. a., 2010. Recombinant immunotherapeutics: current state and perspectives regarding the feasibility and market. Applied microbiology and biotechnology 87, 401-410.

Jozala, A. F., Geraldes, D. C., Tundisi, L. L., de Araújo Feitosa, V., Breyer, C. A., Cardoso, S. L., Mazzola, P. G., de Oliveira-Nascimento, L., de Oliveira Rangel-Yagui, C., de Oliveira Magalhães, P., 2016. Biopharmaceuticals from microorganisms: from production to purification. brazilian journal of microbiology 47, 51-63.

Karst, D. J., Steinhoff, R. F., Kopp, M. R., Serra, E., Soos, M., Zenobi, R., Morbidelli, M., 2017. Intracellular CHO Cell Metabolite Profiling Reveals Steady-State Dependent Metabolic Fingerprints in Perfusion Culture. Biotechnology progress.

Kildegaard, H. F., Baycin-Hizal, D., Lewis, N. E., Betenbaugh, M. J., 2013. The emerging CHO systems biology era: harnessing the 'omics revolution for biotechnology. Current opinion in biotechnology, 1-6.

Kim, D. Y., Chaudhry, M. A., Kennard, M. L., Jardon, M. A., Braasch, K., Dionne, B., Butler, M., Piret, J. M., 2013. Fed-batch CHO cell t-PA production and feed glutamine replacement to reduce ammonia production. Biotechnology progress 29, 165-175.

Kim, W. H., Kim, M. S., Kim, Y.-G., Lee, G. M., 2012. Development of apoptosis-resistant CHO cell line expressing PyLT for the enhancement of transient antibody production. Process Biochemistry 47, 2557-2561.

Kishishita, S., Katayama, S., Kodaira, K., Takagi, Y., Matsuda, H., Okamoto, H., Takuma, S., Hirashima, C., Aoyagi, H., 2015. Optimization of chemically defined feed media for monoclonal antibody production in Chinese hamster ovary cells. Journal of bioscience and bioengineering 120, 78-84.

Konakovsky, V., Clemens, C., Müller, M. M., Bechmann, J., Berger, M., Schlatter, S., Herwig, C., 2016. Metabolic Control in Mammalian Fed-Batch Cell Cultures for Reduced Lactic Acid Accumulation and Improved Process Robustness. Bioengineering 3, 5.

Koumpouras, G., Kontoravdi, C., 2012. Dynamic Optimization of Bioprocesses. 2012, 1487-1495.

Kunaparaju, R., Liao, M., Sunstrom, N.-A., 2005. Epi-CHO, an episomal expression system for recombinant protein production in CHO cells. Biotechnology and bioengineering 91, 670-677.

Kunert, R., Reinhart, D., 2016. Advances in recombinant antibody manufacturing. Applied microbiology and biotechnology 100, 3451-3461.

Lee, J. S., Lee, G. M., 2012. Monitoring of autophagy in Chinese hamster ovary cells using flow cytometry. Methods 56, 375-382.

Lee, K. S., Kang, H. W., Lee, H. T., Kim, H. J., Kim, C. L., Song, J. Y., Lee, K. W., Cha, S. H., 2014. Sequential sub-passage decreases the differentiation potential of canine adipose-derived mesenchymal stem cells. Research in Veterinary Science 96, 267-275.

Li, J., Wong, C. L., Vijayasankaran, N., Hudson, T., Amanullah, A., 2012. Feeding lactate for CHO cell culture processes: impact on culture metabolism and performance. Biotechnology and bioengineering 109, 1173-1186.

Liu, M., Kimura, R., Jayapal, K., Wu, P., Mayer-Bartschmid, A., Trautwein, M., Jockwer, A., 2014. Fed-batch cell culture process development: implementing a novel nutrient additive for a robust, high-titer, scalable process. BioProcess Int 11.

López-Meza, J., Araíz-Hernández, D., Carrillo-Cocom, L. M., López-Pacheco, F., del Refugio Rocha-Pizaña, M., Alvarez, M. M., 2016. Using simple models to describe the kinetics of growth, glucose consumption, and monoclonal antibody formation in naive and infliximab producer CHO cells. Cytotechnology 68, 1287-1300.

McAtee, A. G., Templeton, N., Young, J. D., 2014. Role of Chinese hamster ovary central carbon metabolism in controlling the quality of secreted biotherapeutic proteins. Pharmaceutical Bioprocessing 2, 63-74.

Martínez, J. L., Liu, L., Petranovic, D., Nielsen, J., 2012. Pharmaceutical protein production by yeast: towards production of human blood proteins by microbial fermentation. Current opinion in biotechnology 23, 965-971.

Martínez, V. S., Dietmair, S., Quek, L. E., Hodson, M. P., Gray, P., Nielsen, L. K., 2013. Flux balance analysis of CHO cells before and after a metabolic switch from lactate production to consumption. Biotechnology and bioengineering 110, 660-666.

Mohmad-Saberi, S. E., Hashim, Y. Z. H.-Y., Mel, M., Amid, A., Ahmad-Raus, R., Packeer-Mohamed, V., 2013. Metabolomics profiling of extracellular metabolites in CHO-K1 cells cultured in different types of growth media. Cytotechnology 65, 577-586.

Mosser, M., Kapel, R., Aymes, A., Bonanno, L.-M., Olmos, E., Chevalot, I., Marc, I., Marc, A., 2012. Chromatographic fractionation of yeast extract: A strategy to identify physicochemical properties of compounds promoting CHO cell culture. Process Biochemistry 47, 1178-1185.

Nienow, A. W., Rielly, C. D., Brosnan, K., Bargh, N., Lee, K., Coopman, K., Hewitt, C. J., 2013. The physical characterisation of a microscale parallel bioreactor platform with an industrial CHO cell line expressing an IgG4. Biochemical Engineering Journal 76, 25-36.

Nienow, A. W., Scott, W. H., Hewitt, C. J., Thomas, C. R., Lewis, G., Amanullah, A., Kiss, R., Meier, S. J., 2013. Scale-Down Studies for Assessing the Impact of Different Stress Parameters on Growth and Product Quality during Animal Cell Culture. Chemical Engineering Research and Design. 91, 2265-2274.

Ning, C., 2013. Genome-Based Metabolic Modelling of CHO Cells. (PhD thesis). Imperial College London.

Niu, H., Amribt, Z., Fickers, P., Tan, W., Bogaerts, P., 2013. Metabolic Pathway Analysis and Reduction for Mammalian Cell Cultures - Towards Macroscopic Modeling. Chemical Engineering Science 102, 461-473.

Ozturk, S. S., Riley, M. R., Palsson, B. O., 1992. Effects of ammonia and lactate on hybridoma growth, metabolism, and antibody production. Biotechnology and bioengineering 39, 418-431.

Pan, X., Streefland, M., Dalm, C., Wijffels, R. H., Martens, D. E., 2016. Selection of chemically defined media for CHO cell fed-batch culture processes. Cytotechnology, 1-18.

Pascoe, D. E., Arnott, D., Papoutsakis, E. T., Miller, W. M., Andersen, D. C., Development, B., Way, O. D. N. A., Francisco, S. S., 2007. Proteome Analysis of Antibody-Producing CHO Cell Lines With Different Metabolic Profiles. 98, 391-410.

Reinhart, D., Damjanovic, L., Kaisermayer, C., Kunert, R., 2015. Benchmarking of commercially available CHO cell culture media for antibody production. Applied microbiology and biotechnology 99, 4645-4657.

Richardson, J., Shah, B., Bondarenko, P. V., Bhebe, P., Zhang, Z., Nicklaus, M., Kombe, M. C., 2015. Metabolomics analysis of soy hydrolysates for the identification of productivity markers of mammalian cells for manufacturing therapeutic proteins. Biotechnology progress 31, 522-531.

Robitaille, J., Chen, J., Jolicoeur, M., 2015. A single dynamic metabolic model can describe mAb producing CHO cell batch and fed-batch cultures on different culture media. PloS one 10, e0136815.

Sani, M., 2015. Evaluation of microwell based systems and miniature bioreactors for rapid cell culture bioprocess development and scale-up. (PhD thesis). University College London.

Scott, W. H., 2011. The effect of potential large-scale bioreactor environmental heterogeneities during fed-batch culture on the performance of an industrially-relevant GS-CHO cell culture, producing an IgG antibody. (PhD thesis). University of Birmingham.

Sellick, C. A., Croxford, A. S., Maqsood, A. R., Stephens, G., Westerhoff, H. V., Goodacre, R., Dickson, A. J., 2011. Metabolite profiling of recombinant CHO cells: designing tailored feeding regimes that enhance recombinant antibody production. Biotechnol Bioeng 108, 3025-3031.

Sellick, C. A., Croxford, A. S., Maqsood, A. R., Stephens, G. M., Westerhoff, H. V., Goodacre, R., Dickson, A. J., 2015. Metabolite profiling of CHO cells: Molecular reflections of bioprocessing effectiveness. Biotechnology journal 10, 1434-1445.

Sellick, C. a., Hansen, R., Maqsood, A. R., Dunn, W. B., Stephens, G. M., Goodacre, R., Dickson, A. J., 2009. Effective quenching processes for physiologically valid metabolite profiling of suspension cultured mammalian cells. Analytical Chemistry 81, 174-183.

Sellick, C. a., Knight, D., Croxford, A. S., Maqsood, A. R., Stephens, G. M., Goodacre, R., Dickson, A. J., 2010. Evaluation of extraction processes for intracellular metabolite profiling of mammalian cells: Matching extraction approaches to cell type and metabolite targets. Metabolomics 6, 427-438.

Selvarasu, S., Ho, Y. S., Chong, W. P. K., Wong, N. S. C., Yusufi, F. N. K., Lee, Y. Y., Yap, M. G. S., Lee, D.-Y., 2012. Combined in silico modeling and metabolomics analysis to characterize fed-batch CHO cell culture. Biotechnology and bioengineering 109, 1415-1429.

Shimura, Y., Suzuki, J., Kasuya, M. C. Z., Matsuoka, K., Hatanaka, K., 2012. Influence of passage number on glycosylation of alkyl lactosides by Madin-Darby canine kidney (MDCK) cells. Journal of bioscience and bioengineering 114, 552-555.

Sieck, J. B., Cordes, T., Budach, W. E., Rhiel, M. H., Suemeghy, Z., Leist, C., Villiger, T. K., Morbidelli, M., Soos, M., 2012. Development of a Scale-Down Model of hydrodynamic stress to study the performance of an industrial CHO cell line under simulated production scale bioreactor conditions. Journal of biotechnology 164, 41-49.

Simon, A. E., Taylor, M. W., Bradley, W. E., Thompson, L. H., 1982. Model involving gene inactivation in the generation of autosomal recessive mutants in mammalian cells in culture. Molecular and cellular biology 2, 1126-1133.

Sokolov, M., Ritscher, J., MacKinnon, N., Bielser, J. M., Brühlmann, D., Rothenhäusler, D., Thanei, G., Soos, M., Stettler, M., Souquet, J., 2017. Robust factor selection in early cell culture process development for the production of a biosimilar monoclonal antibody. Biotechnology progress 33, 181-191.

Steinhoff, R. F., Karst, D. J., Steinebach, F., Kopp, M. R., Schmidt, G. W., Stettler, A., Krismer, J., Soos, M., Pabst, M., Hierlemann, A., 2016. Microarray-based MALDI-TOF mass spectrometry enables monitoring of monoclonal antibody production in batch and perfusion cell cultures. Methods 104, 33-40.

Tescione, L., Lambropoulos, J., Paranandi, M. R., Makagiansar, H., Ryll, T., 2015. Application of bioreactor design principles and multivariate analysis for development of cell culture scale down models. Biotechnology and bioengineering 112, 84-97.

Toussaint, C., Henry, O., Durocher, Y., 2016. Metabolic engineering of CHO cells to alter lactate metabolism during fed-batch cultures. Journal of biotechnology 217, 122-131.

Tuñón, J., Martín-Ventura, J. L., Blanco-Colio, L. M., Lorenzo, Ó., López, J. A., Egido, J., 2010. Proteomic strategies in the search of new biomarkers in atherothrombosis. Journal of the American College of Cardiology 55, 2009-2016.

van Veen, H. a., Koiter, J., Vogelezang, C. J. M., van Wessel, N., van Dam, T., Velterop, I., van Houdt, K., Kupers, L., Horbach, D., Salaheddine, M., Nuijens, J. H., Mannesse, M. L. M., 2012. Characterization of recombinant human C1 inhibitor secreted in milk of transgenic rabbits. Journal of biotechnology 162, 319-326.

Volmer, M., Northoff, S., Scholz, S., Thüte, T., Büntemeyer, H., Noll, T., 2011. Fast filtration for metabolome sampling of suspended animal cells. Biotechnology Letters 33, 495-502.

Wagstaff, J. L., Masterton, R. J., Povey, J. F., Smales, C. M., Howard, M. J., 2013. 1H NMR Spectroscopy Profiling of Metabolic Reprogramming of Chinese Hamster Ovary Cells upon a Temperature Shift during Culture. PLoS ONE 8, 1-14.

Walsh, G., 2014. Biopharmaceutical benchmarks 2014. Nature biotechnology 32, 992-1000.

Wang, Z., Park, J. H., Park, H. H., Tan, W., Park, T. H., 2010. Enhancement of therapeutic monoclonal antibody production in CHO cells using 30Kc6 gene. Process Biochemistry 45, 1852-1856.

Wen, Y., Zang, R., Zhang, X., Yang, S.-T., 2012. A 24-microwell plate with improved mixing and scalable performance for high throughput cell cultures. Process Biochemistry 47, 612-618.

Xiao, Z., Sabourin, M., Piras, G., Gorfien, S. F., 2014. Screening and Optimization of Chemically Defined Media and Feeds with Integrated and Statistical Approaches. In: Pörtner, R. (Ed.), Animal Cell Biotechnology: Methods and Protocols. Humana Press, Totowa, NJ, pp. 117-135.

Xu, P., Clark, C., Ryder, T., Sparks, C., Zhou, J., Wang, M., Russell, R., Scott, C., 2017. Characterization of TAP Ambr 250 disposable bioreactors, as a reliable scale-down model for biologics process development. Biotechnology progress 33, 478-489.

Zang, L., Frenkel, R., Simeone, J., Lanan, M., Byers, M., Lyubarskaya, Y., 2011. Metabolomics profiling of cell culture media leading to the identification of riboflavin photosensitized degradation of tryptophan causing slow growth in cell culture. Analytical chemistry 83, 5422-5430.

Zhang, A., Tsang, V. L., Moore, B., Shen, V., Huang, Y. M., Kshirsagar, R., Ryll, T., 2015. Advanced process monitoring and feedback control to enhance cell culture process production and robustness. Biotechnology and bioengineering 112, 2495-2504.

Zhang, H., Wang, H., Liu, M., Zhang, T., Zhang, J., Wang, X., Xiang, W., 2013. Rational development of a serum-free medium and fed-batch process for a GS-CHO cell line expressing recombinant antibody. Cytotechnology 65, 363-378.

Zhou, M., Crawford, Y., Ng, D., Tung, J., Pynn, A. F. J., Meier, A., Yuk, I. H., Vijayasankaran, N., Leach, K., Joly, J., Snedecor, B., Shen, A., 2011. Decreasing lactate level and increasing antibody production in Chinese Hamster Ovary cells (CHO) by reducing the expression of lactate dehydrogenase and pyruvate dehydrogenase kinases. Journal of biotechnology 153, 27-34.

Zhu, J., 2012. Mammalian cell protein expression for biopharmaceutical production. Biotechnology advances 30, 1158-1170.