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Study and characterisation of human HEK293 cell line as a platform for recombinant protein production

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CERTIFIQUEM:

Que la llicenciada Leticia Liste Calleja ha dut a terme sota la nostra direcció, en els laboratoris del Departament d'Enginyeria Química, el treball que amb el títol de: "**Study and characterisation of human HEK293 cell line as a platform for recombinant protein production**" es presenta en aquesta memòria, la qual constitueix la seva Tesi per optar al grau de Doctor en Biotecnologia.

I perquè en prengueu coneixement I tingui els efectes que correspongui, presentem davant de l'Escola de Doctorat de la Universitat Autònoma de Barcelona l'esmentada Tesi signant aquesta certificació a

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SUMMARY

The thesis is focused on the study of recombinant protein production in mammalian cell lines. In particular, the study of three different approaches of different bioprocesses based on HEK293 cells has been addressed. As a model protein for recombinant expression, CapPCV2 has been selected. This protein makes up the viral capsid of Porcine circovirus serotype 2 (PCV2), which is the causative agent of PCVDs (porcine circovirus diseases), a group of diseases with major impact in pig's industry worldwide. This project has been addressed from the perspective of bioprocess development and optimization and therefore, the increment of volumetric production of cells, virus and proteins have been the driving force of the research.

Firstly, cell culture media and nutritional supplementation studies are presented. Cell growth relies in high extent to the nutritional and physicochemical characteristics of the media in which cells are cultured and therefore, finding the proper cell media is one of the key factors for cell culture expansion. The initial media study resulted in a 6-fold increment of the maximal viable cell achieved in the original media. Besides, different cell culture strategies have been explored, which resulted in a fed-batch strategy that allowed reaching maximal viable cell densities of $26.8x10^6$ cell/mL, which represents 13-fold increment on maximal viable cell density originally reached.

In the second and third chapter of results, three different approaches for the expression of recombinant CapPCV2 (r-CapPCV2) are evaluated and discussed. As a first approach, a viral recombinant adenovirus encoding for the gene *Cap*PCV2 has been generated and used as viral vector for the production of the recombinant protein in HEK293 cells. Besides, a deep study of the main parameters that affect the infection performance has been carried out and discussed in order to find the best media, MOI (multiplicity of infection), TOI (time of infection) and TOH (time of harvest) for adenovirus and recombinant protein production. This study was performed with an adenovirus expressing the reporter gene GFP and thereafter, the best infection parameters encountered were applied for the production of r-CapPCV2 (media: SFMTransFx-293 supplemented with 4mM glutaMAX, 5% FBS and 10%CB5; MOI:1; TOI:$1x10^6$ cell/mL) and TOH:48hpi). The second and third strategies are both based on the generation of stable producer cell lines, but one strategy relies on illegitimate (or random) integration of the gene in the HEK293 genome ,whereas the other strategy is a site-directed integration of the

gene in previously characterized hot-spots (i.e. high-active transcribed regions from genome). The site-directed integration was performed using RMCE technology (Recombinant mediated cassette exchange). After the comparison of the specific and volumetric productivities achieved with each approach, the best producer has been selected. Nevertheless, r-CapPCV2 was poorly produced so it was unfeasible to develop/design a cost-effective industrial bioprocess and other alternatives must be studied in the future.

Finally, the study of an unexpected metabolic behaviour observed in HEK293 cells cultured in our lab has been addressed from a physiologic and metabolic perspective. HEK293 cells could concomitantly consume glucose and lactate in exponentially growing cultures at particular environmental conditions. After a deep study of these conditions, it was found out that the switch from lactate secretion (which is the main drawback of mammalian high cell density cultures) to lactate consumption can be triggered from the beginning of cell culture at pH_0=6.6 together with the addition of 4-12mM of lactate to media. Remarkably, under these conditions nor cell growth neither protein production were negatively affected. Form these results, we hypothesize that HEK293 can co-transport lactate and H^+ to the cytosol as a pH-detoxification mechanism. Moreover, the application of flux balance analysis permitted to find out that when lactate and glucose are consumed together a "more balanced" metabolism is achieved, meaning that glycolytic and TCA fluxes became similar, avoiding pyruvate accumulation at the cytosol and consequently, lactate formation. This is totally opposed to the extensively observed metabolism of exponentially growing mammalian cell lines, where the high flux through the glycolytic pathway encounters a limitation on the fluxes entering the mitochondria (hence, the TCA cycle) and consequently lactate is produced and secreted to media. The construction of a HEK293 metabolic model and the application of FBA will allow making in silico predictions of metabolic beahaviours after the upregulation or downregulation of target genes. This strategy may open the possibility of generate engineered HEK293 cell lines with an optimised metabolism in order to study more efficient cell culture strategies towards the achievement of higher cell densities and product titres.

RESUM

El present treball es centra en l'estudi de la producció de proteïnes recombinants en línies cel·lulars de mamífer. Concretament, s'ha realitzat l'estudi de tres estratègies de bioprocés, totes elles basades en el cultiu de cèl·lules HEK293. Com a proteïna model per a l'expressió de proteïnes heteròlogues s'ha triat la proteïna CapPCV2, la qual conforma la càpsida viral del Circovirus porcí serotip 2 (PCV2). Aquest virus és l'agent causal de PCVDS (porcine circovirus diseases o malaties derivades de circovirus porcí). Aquest terme engloba un conjunt de malalties i síndromes que tenen un elevat impacte econòmic en la indústria porcina. El projecte s'ha enfocat des de la perspectiva de desenvolupament i optimització del bioprocés i, en conseqüència, l'increment de la producció volumètrica ha estat la força impulsora de tot el treball.

En primer lloc es presenten els estudis per a la selecció del medi de cultiu i suplements nutricionals. El creixement cel·lular depèn en gran mesura de les característiques nutricionals i fisicoquímiques del medi en que se les cultiva. Per tant, trobar el medi adequat és un dels factors clau per a l'expansió del cultiu cel·lular. L'estudi inicial de medis de cultiu va permetre augmentar sis vegades la densitat de cèl·lules viables en comparació al medi original en que es cultivaven. D'altra banda, s'han explorat diferents estratègies de cultiu, i com a resultat s'ha implementat una estratègia de fed-batch que ha permès arribar a densitats cel·lulars de $26.8x10^6$ cell/mL.

En el segon i tercer capítol de resultats, s'avaluen tres estratègies diferents per a la producció de la proteïna recombinant CapPCV2 (r-CapPCV2). La primera estratègia ha estat la infecció de cèl·lules HEK293 amb un vector adenoviral que codifica el gen de la CapPCV2 (vector generat dins del treball d'aquesta tesis doctoral). Els paràmetres d'infecció s'han estudiat en profunditat per tal de trobar els paràmetres d'infecció (medi de cultiu, MOI (multiplicitat d'infecció), TOI (temps d'infecció) i TOH (temps de recollida)) per a la millora de la producció de la proteïna i el vector adenoviral. La segona i tercera estratègia estan basades en la generació de línies cel·lulars estables. Concretament, s'ha generat una línia cel·lular productora de r-CapPCV2 a partir de la integració a l'atzar del vector plasmídic en el genoma de la cèl·lula. D'altra banda, s'han generat línies cel·lulars amb la integració dirigida del gen en llocs prèviament caracteritzats com d'altra transcripció genètica. La integració dirigida s'ha

efectuat mitjançant la tecnologia RMCE (recombinant mediated cassette exchange, o bescanvi de casset mitjançada per recombinació). Després de la comparació de les productivitats específiques i volumètriques aconseguides amb cada estratègia, el millor productor va ser seleccionat. Nogensmenys, r-CapPCV2 es produeix en quantitats molt baixes i per tant no ha sigut possible dissenyar un procés de producció rentable i altres alternatives de producció s'haurien d'estudiar en un futur.

Finalment, l'estudi d'un comportament metabòlic particular observat en les cèl.lules en cultiu s'ha adreçat des d'una perspectiva fisiològica i metabòlica. A certes condicions extracel·lulars, s'ha observat que les cèl·lules HEK293 poden consumir de manera simultània glucosa i lactat durant el seu creixement exponencial. Després d'un ampli estudi d'aquestes condicions, s'ha determinat que el canvi de la producció d'àcid làctic (que és el principal problema dels cultius d'alta densitat de cèl·lules de mamífer) cap al consum d'aquest metabòlit pot ser generat des de el començament del cultiu quan el pH és de 6.6 i la concentració de lactat és de 4-8mM. En aquestes condicions, ni el creixement cel·lular ni la producció de proteïna es veuen afectades negativament. A la llum d'aquests resultats, es genera la hipòtesi de que les cèl·lules HEK293 poden co-transportar el lactat extracel.lular i els protons com un mecanisme de detoxificació del pH. D'altra banda, l'aplicació de l'anàlisi de balanç de fluxos (FBA) ha revelat que quan la glucosa i el lactat es consumeixen simultàniament s'aconsegueix un metabolisme "equilibrat", és a dir els fluxos de la glicòlisi i el cicle TCA esdevenen similars, evitant l'acumulació de piruvat en el citosol, la seva transformació a làctic i finalment la secreció d'aquest metabòlit. Aquest comportament és totalment oposat al que s'observa de forma general en els cultius de cèl.lules de mamífer en creixement exponencial, on els elevats fluxos de la glicòlisi troben una limitació en els fluxos d'entrada a la mitocòndria (és a dir, del cicle TCA) i conseqüentment el lactat és produït i secretat al medi. La construcció d'un model metabòlic i l'aplicació de FBA permetrà fer prediccions in silico de comportaments metabòlics causats per la sobreexpressió o el silenciament de gens diana. Aquesta estratègia obre la possibilitat de generar línies cel·lulars que presentin un metabolisme optimitzat per tal d'estudiar estratègies de cultiu més eficients per a l'increment de la densitat cel·lular i productivitat de proteïna recombinant.

NOMENCLATURE

$[IPU]_0$	Infective particle unit concentration at the time of infection
$[IPU]_f$	Infective particle unit concentration at the time of harvest
$[lac]_0$	Initial lactate concentration
ADCF	Animal derived component free
AdV	Adenovirus
CapPCV2	capside protein of porcine circovirus serotype 2
*Cap*PCV2	Gene of the capside of PCV2
CapPCV2	Protein of the capside of PCV2
*Cap*PCV2op	Gene of the capside of PCV2, DNA sequence optimized for its expression in HEK293 cells
CB5	Cell Boost 5
CPE	cytopathic effect
dneo or *Δneo*	Neomycin resistance gene, ATG codon deleted
dpi	days post infection
dpt	days post transfection
FBS	Fetal bovine serum
FU	Fluorescence units
GFP	Green fluorescence protein
GFP	Green fluorescence protein
GOI	Gene of interest
GOI	Gene of interest
HCDC	High cell density cultures
hpi	hours post infection
hpt	hours post transfection
IPU	Infective particle unit
IPU_R	Infective particle unit ratio
MOI	Multiplicity of infection
neo	Neomycin resistance gene
PCV2	Porcine circovirus serotype 2
pH_0	Initial pH value
POI	product of interest
pTag	Tagging vector
pTar	Targeting vector
q_{fluo}	Specific fluorescence rate
q_{GFP}	Specific GFP production rate
Qp_{AdV}	Volumetric AdV production
Qp_{GFP}	Volumetric GFP productivity
r-CapPCV2	recombinant protein of CapPCV2
rAdV	recombinant adenovirus
rAdV-*Cap*PCV2	recombinant adenovirus encoding for CapPCV2 gene
RMCE	Recombinase mediated cassette exchange

SSIL2	Secretion signal of Interleukin-2
TOH	Time of harvest
TOI	Time of infection
$t_{\mu max}$	Exponential growth phase (or time of μ_{max})
Vp_{AdV}	Volumetric AdV production
Vp_{GFP}	Volumetric GFP production
Xv	Viable Cell density
Xv_{max}	Maximal viable cell density
$Y_{AdV/X}$	Specific AdV production
$Y_{CapPCV2/X}$	Specific CapPCV2 production
$Y_{GFP/X}$	Specific GFP production
Δlac	Lactate accumulated in extracellular media
μ	Specific growth rate
μ_{max}	Maximal specific growth rate
$\mu_{post\ ad}$	Specific growth rate after the adition of a component to cell media

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CHAPTER 1. GENERAL INTRODUCTION

1. 1. BIOTECHNOLOGICAL PROCESSES: BIOPROCESSES

A bioprocess is a specific process that uses complete microorganisms, partial fraction of microorganisms biological systems or molecules (e.g. virus and enzymes) to obtain new products or to offer a service (environmental or grey biotechnology). Bioprocesses are related to different types of industry such as food, chemical, environmental and veterinarian or human pharmaceutical industry, being the latter an outstanding and active sector. Among the wide range of existing industrial bioprocesses, it is of important relevance the use of microorganisms for recombinant protein production. It has been more than thirty years since the first recombinant protein was obtained and the industrial production of recombinant products became possible. Since then, hundreds of proteins from very different origins have been produced by genetically modified organisms[1]. Recombinant products have reached the market in diverse areas, including pharmaceutical, veterinary food, pesticides and detergents. Noteworthy, approximately 30 recombinant products in the pharmaceutical sector account for more than 90 per cent of all recombinant product sales and seven of these proteins alone own approximately 70 per cent of the total market: erythropoietin, alpha interferon, hepatitis-B vaccine granulocyte colonoy stimulating factor, insulin, human growth hormone and tissue plasminogen activator (tPA)[1]. Hence, biopharmaceuticals are considered the most profitable recombinant proteins produced nowadays.

1.1.1. BIOPHARMACEUTICALS PRODUCTS: VACCINES

Among the biopharmaceuticals currently studied and produced, we can find several categories: (1) cytokines, (2) enzymes, (3) hormones, (4) clotting factors, (5) vaccines, (6) monoclonal antibodies, (7) cell therapies, (8) antisense drugs and (9) peptide therapeutics. Particularlly, the present research project is focused on the generation of a platform for production of vaccines candidates using a human cell line as a biocatalizer (HEK293).

Vaccines can be classified in two groups depeding on its usage: as a desease preventive protection (prophylaxis) or as a therapeutic agent. Nowa days, prophylaxis is by far the most extended use of vaccination, whereas therapeutic vaccines are currently investigated.
The use of vaccines in prophyilaxis is the most effective method of preventing infectious diseases. Widespread immunity due to vaccination is largely responsible for the worldwide eradication of smallpox and the restriction of diseases such as polio, measles, and tetanus from much of the world. The first description of a protective effect of a vaccination was observed in 1798 by Edward Jenner, who described the protective effect of cowpox against smallpox[2].

Vaccines bring economic benefits beyond health. Vaccines avert illness both directly through immunization and indirectly through herd immunity effect (i.e. the reduction of infection or disease in the unimmunized segment as a result of immunizing a proportion of the population). Therefore, vaccination strategy might avoid the expense on treatment of the particular disease, which in some cases can be high due to disease duration or effects. The high efficiency of vaccines and their social benefits has pushed forward their development and commercialization. In fact, there are some economical studies which report that although the market share of vaccines among the drug sales is quite small (2-3% of the global pharmaceutical market), it has been increasing at a spectacular growth rate over the last years (10-15% increment per year versus 5-7% for pharmaceuticals)[3].

The overall vaccine research and developmental approach can be broadly categorized into three generations (Figure 1.1): (1) the first generation of vaccine development was essentially based on the basic principles of Louis Pasteur, which consist of using inactivated pathogens in whole or live attenuated forms as vaccine[4,5]; (2) the second generation vaccines are made up of purified cell components (referred as subunit vaccines)[6]. This approach has been more recently exploited with the recombinant DNA technology, which has enabled the production of antigenic proteins in non-natural host for a particular pathogen. In the last decade, the discovery that monomeric antigenic

proteins can self-assemble into virus like particles (VLPs) has raised even more the research efforts and the economic impact of second generation vaccines[7–9]. (3) The third generation vaccines are still in the experimental phase. They are vaccine in which either 'naked DNA' is injected directly to produce an immunological response, or packaged in a recombinant virus or bacteria.

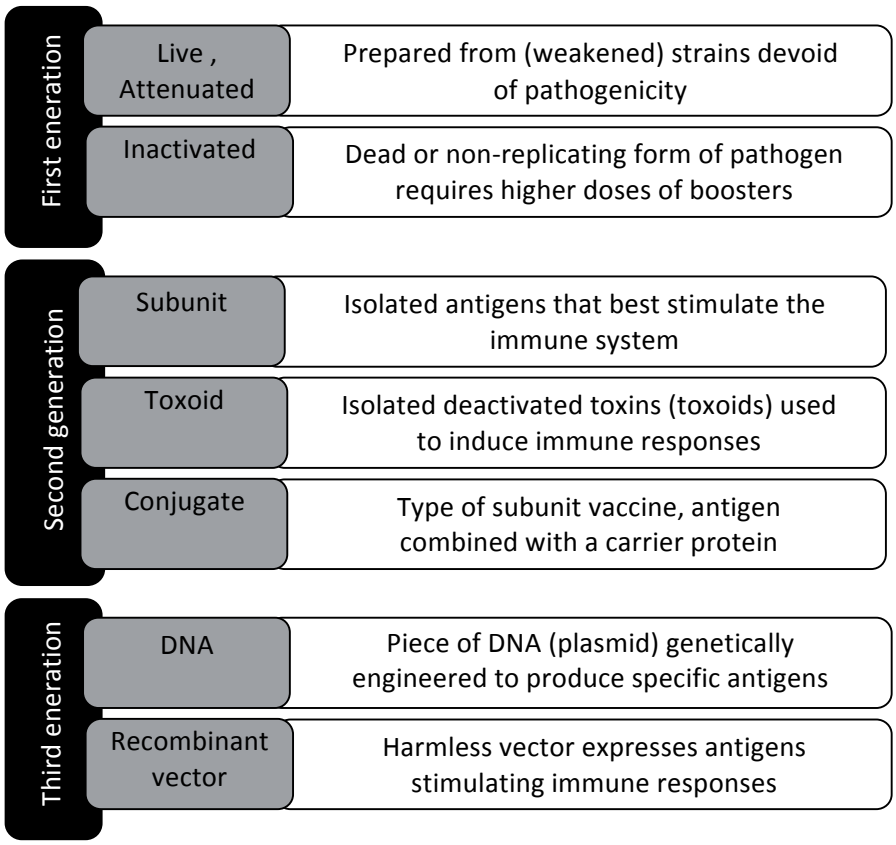

Figure 1.1. Classification of vaccines by technology. Adpated from[10].

Vaccines market is usually differentiated between human vaccines and veterinarian vaccines. The criteria for successful veterinary vaccines can be very different from those for human vaccines depending on the animal groups under consideration. For example, criteria for companion animal vaccines are similar to those for human vaccines in that the health and welfare of the individual animal are primary concerns. The main objectives of vaccines for livestock immunization purpouses are the improvement of the overall production for the primary producers and the cost-benefit resulting from vaccination is the bottom line for this industry.

Focusing on the veterinary vaccines is worth to mention that the selling price of such vaccines is much lower in comparison to the human vaccines. The lower vaccines price is compensated somehow by the lower stringent regulatory and preclinical trial requeriments in the veterinary

sector, but the optimization of vaccine production bioprocesses is of huge importance in order to define a profitable manufacturing bioprocess.
In the present research thesis, a bioprocess based on HEK293 cells for the generation of second generation vaccine intended for veterinarian purposes has been explored.

1.2. BIOPROCESS DEVELOPMENT AND OPTIMIZATION

Once the product of interest has been selected, the bioprocess development starts with the selection of the producer organism, which will be mainly defined by the targeted product itself. Though all organisms show advantages and disadvantages (as can be seen in Figure 1.2), in this work mammalian cells were the final option of choice. Reasons for this selection are exposed in point 1.3.1.

Once the biocatalizer has been selected, the study performed in this thesis about bioprocess development aimed to optimize such bioprocess. Scientists and engineers have traditionally tackled the attemps of maximizing productivity and minimizing costs on the production process from three main perspectives: genetic engineeting, bioprocess engineering and metabolic engineering. All three have been considered in this research project following different strategies. The key information taken into account for designing and developing these strategies is also explained in the following points.

1.2.1. MAMMALIAN CELLS FOR RECOMBINANT PROTEIN PRODUCTION

Microbial sistems can be considered an attractive option for expressing certain biopharmaceutical proteins due to their low cost, high productivity, and rapid implementation. Moreover, there is no adventitious virus concern to regulatory authorities. In fact, relatively simple recombinant proteins, such as insulin[11,12] and bovine growth hormone[11], have been successfully produced in *Escherichia coli* or *Saccharomyces cerevisiae*. However, many biopharmaceutical molecules are too large and complex to be made by simple prokaryotic bacteria, or even the lower eukaryotic forms such as fungi and yeasts. Complex biomolecules, such as functional MAbs or highly glycosylated proteins, require the posttranscriptional metabolic machinery only available in mammalian cells[12]. Proper posttranslational modifications convey higher quality and efficacy to the protein when compared

with proteins produced by microbes. Aglycosylated forms of glycoproteins tend to be misfolded, biologically inactive, or rapidly cleared from circulation[13]. Eukaryotic fungi and yeast can glycosylate

	E. coli	Yeast	Insect cells	Mammalian cells
Cell growth	Rapid	Rapid	Slow	Slow
Cost of medium	Low	Low	High	High
Expression level	High	Low-high	Low-high	Low-moderate
Post-translational modifications				
Protein folding	Refolding usually	Refolding sometimes	Proper folding	Proper folding
N-linked glycosylation	None	High mannose	Simple, no sialic acid	Yes
O-linked glycosylation	✗	✓	✓	✓
Phosphorylation	✗	✓	✓	✓
Acetylation	✗	✓	✓	✓
Acylation	✗	✓	✓	✓
Gamma-carboxylation	✗	✗	✗	✓

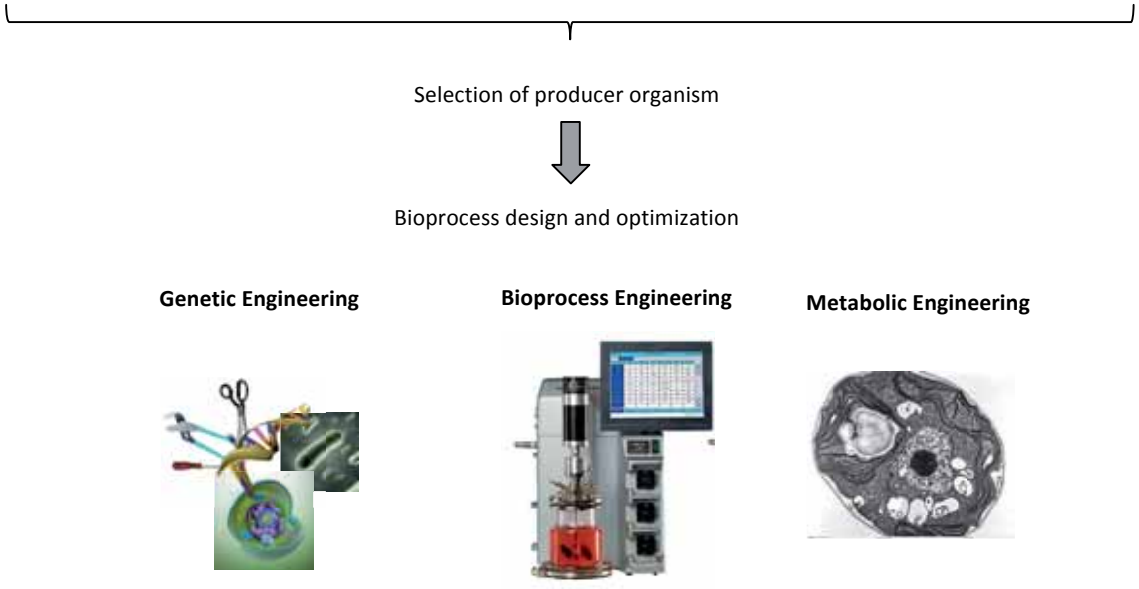

Figure 1.2. Scheme of the main areas to be considered during bioprocess development and optimization.

proteins, but the resultant glycans are not the structures normally found on human proteins[14]. Regarding the eukaryotic plant cells, they are hardly used in biopharmaceuticals field. Protein production in these cells typically results in the formation of a hyperglycosylated product, often containing sugar molecules immunogenic in humans. Besides, the resulting proteins generated in plant-cell systems, often present reduced timelife in serum[15]. As for the eukaryotic insect cells, they possess only limited glycosylation capabilities and are mostly used as vehicles for production of genetically engineered viral vaccines using baculovirus expression systems[16]. For all these reasons, mammalian cells are the preferred hosts for the production of most complex protein therapeutics, since their functionally and pharmacokinetically relevant posttranslational modifications are highly human compatible.

In fact, as the need for producing properly folded protein molecules with appropriate glycosylation patterns grew, the industry shifted in the 1980s towards the use of mammalian cells (Figure 1.3). The economic concern about low productivities from mammalian cells has been eased by the 1- to 2-g/liter yield commonly achieved and 10-g/liter yield recently claimed on the production scale[17]. Therefore, during the past 2 decades, despite the well-known lower production yield higher manufacturing cost, and other challenges compared with microbial systems, cultured mammalian cells have become a widely used platform for producing recombinant proteins [18].

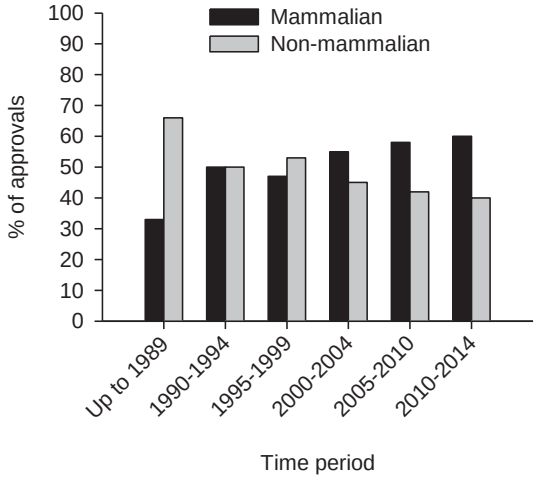

Figure 1.3. Relative application of mammalian versus nonmammalian-based expression systems in the production of biopharmaceuticals over the indicated time-periods. Adapted from [15].

1.2.1.1. THE BIOCATALYST: HEK293 CELL LINE.

There are several established cell lines for recombinant protein production. The most widely used in industrial bioprocesses are CHO, NS0 and Sp2/O-Ag14. Nevertheless, HEK293 cell line has been gaining importance during the past decade. This particular line was initiated by the transformation and culturing of normal HEK cells with sheared adenovirus 5 DNA. The transformation resulted in the incorporation of approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells[19]. The incorporated viral DNA sequence encodes for E1A and E1B genes, which are essential for adenovirus replication. The increasing use of HEK293 cell line over the years is mainly due to two reasons:

1. **The need of recombinant adenovirus production for their use in gene therapy, as well as live viral vaccines.**

   The transformed human embryonic kidney cell line 293 can support the replication of an

E1A + E1B-defective adenoviral particle[19]. These vectors are basically wild-type human adenoviruses in which the E1A and E1B, the essential genes, have been deleted. According to a recent review[20] from the first gene therapy trial approval on 1989, an increasing tendency on approved trials occurred until a maximum of 116 on 1999. Thereafter, a slight decrement was noticed until 2003 due to a few unfortunate cases of serious adverse events[21], followed by a new regrowth of the interest in this kind of therapy until 2006. In the same outstanding review, it is reported that along with the increasing interest on gene therapy, adenoviral vectors have become the most commonly used vectors (Figure 1.4), making necessary the increase of its production (hence, use of HEK293 cells) over the last decades. Besides, their usefulness for permanent gene replacement is limited by their high immunogenicity, which has resulted in rapid elimination of transduced cells through induction of T and B cells to antigens of AdV and the transgene product. Due to this aptitude for inducing potent innate and adoptive immune responses, Ad vectors have been and are

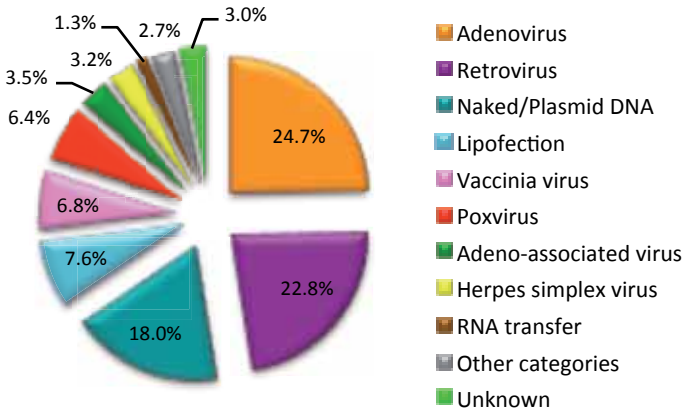

Figure 1.5. Gene therapy vectors used in clinical trials. Adapted from[20]

being explored as vaccine carriers (Table 1.1)[22], which also increase the necessity of the optimization and increment of their production.

Table 1.1. Examples of adenovirus vectors used for vaccination. Adapted from[22]

Ad Serotype	Pathogen/Disease	Stage of development	Ad Serotype	Pathogen/Disease	Stage of development
AdHU5	Pneumococcus	Mice	AdHU5	Malaria	Mice
AdHU5	Botulism	Mice	AdHU26	Simian immunodeficiency virus	Rhesus macaques[a]
AdHU5	Venezuelan encephalopatitis virus	Mice	AdHU35	CEA/cancer	Mice
AdHU5	Japanese encephalopatitis virus	Mice	AdC3	Rabies	Mice
AdHU5	Malaria	Mice[a]	AdC68	Human papillomavirus	Mice
AdHU5	Alzheimer	Mice	AdC6	Human immunodeficiency virus	Rhesus macaques
AdHU5	Dengue	Rhesus macaques	AdC6, Ad7, Ad9	Malaria	Mice
AdHU5	Influenza virus (H5N1)	Mice			

Abbreviations: Ad, adenovirus; CEA, carcinoembryonic antigen.
[a]Phase 1 human trials ongoing

2. The high capacitiy of transfection of HEK293 cell line.

As the first step on the generation of stable cell lines for recombinant protein production is the transfection of the cell line with the vector encoding for the gene of interest, it is highly valuable to get a cell line in which this first step is easy to accomplish. Besides, the interest on transient gene expression for large-scale recombinant protein prodcution has increased over the past decades. Although stable cell lines continue to be the most desired strategy for protein production, in some cases faster approaches to recombinant protein production are preferred (e.g. when one must rapidly evaluate many candidate proteins or when several variants of a single protein need to be studied for their potential as biologics)[23]. Early attempts to employ the same protocols for the production of recombinant proteins in milligram to gram quantities on a larger scale focused on the use of COS cell lines [1–4], but were of limited success due to the restricted scalability of this naturally very adherent cell line. Furthermore, the relatively short life-span of the transfected culture rendered the approach less valuable. A major breakthrough in transient gene expression (TGE) technologies was achieved by switching to other host cell lines, such as HEK293[24].

A series of genetically modified or clonally selected HEK293 descendants have been developed commercially or in academia (Table 1.2). While 293SF-3F6 cells are a derivative from wildtype strain

selected for serum-free suspension culture and adenovirus vector high productivity levels[25], most of the other derivatives are actually engineered cell lines. These cell lines feature genetic elements such as the EBNA-1 gene of the Epstein–Barr virus or the SV40 large T-antigen of the SV40 virus which, in conjunction with their origins of replication (oriP and SV40ori, respectively), when provided in trans are claimed to boost protein expression by stable episomal maintenance and replication of the expression plasmids. Additionally, these elements enhance the nuclear import of expression vectors by means of nuclear targeting sequences present within the 72-bp-enhancer element of SV40. Stable insertion of the EBNA-1 gene as well as the SV40 large T-antigen genes via antibiotic selection has rendered the respective HEK.EBNA and HEK293 T cell lines Geneticin (G-418) resistant[24].

In the present work, the original cell line, the HEK293-3F6 and the HEK293T derivatives were used for the production of the recombinant protein of interest.

Table 1.2. Overview of current HEK293 cell lines. Adapted from[24].

Expression system	Features of cell line	Cultivation medium*
293 Freestyle (293-F)	HEK293 wild-type	Suspension: Freestyle medium (Invitrogen)
HEK.EBNA (HE, 293-EBNA)	EBNA-1 transformed HEK293 cell line	Suspension: e.g. ExCell 293 (SAFC Biosciences), Freestyle 293 (Invitrogen)
293-SFE (293SF-2f6, NRC)	Suspension-adapted EBNA-1 transformed 293 cell line	Suspension: hybridoma serum-free medium+1%BCS
HEK293T	Sv40 T-antigen transformed 293 cell line	Adherent: DMEM+10% FCS, suspension not routinely done
HKB-1 1 (Hybrid of Kidney and B-cell)	Fusion of 293 cell with B-cell lymphoma cell line	Suspension: Bayer proprietary medium

*Cultivation medium indicated is as found in literature, but it does not exclude that the particular cell line could be cultivated in other medium.

1.2.2. RECOMBINANT DNA ENGINEERING

Genetic engineering, also called genetic modification, is the direct manipulation of an organism's genome using biotechnology. Foreign DNA can be inserted in the host genome using many different cloning strategies.

In molecular cloning, a vector is a DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and/or expressed. A vector containing foreign

DNA is called recombinant DNA. The four major types of vectors are plasmids, viral vectors, cosmids, and artificial chromosomes. The first two ones are the ones used in this doctoral thesis:

1.2.2.1. VIRAL VECTORS FOR RECOMBINANT PROTEIN EXPRESSION

An easy and very effective way of delivering the gene of interest is through viral vectors. Viruses have evolved to deliver their genetic material to their host in an efficient and non-destructive way. Some viral vectors, such as retroviruses, promote integration of the viral genome into the cell's chromosome. Many others are used for transient expression. In these cases, recombinant protein production occurs only during certain stages of the life cycle of the virus. Common viral vectors are summarized in Table 1.3. The simplicity of virus-driven protein expression makes it useful for production in higher eukaryotes, as obtaining stable recombinant animal cells may be a tedious and long procedure.

Table 1.3. Summary of the main characteristics of the most common viral vectors. Adapated from[26].

	Adenovirus	**Adeno-associated virus**	**Retrovirus**	**Herpes virus**	**Poxvirus (vaccinia)**	**Alfavirus**
Maximal length of transgene	30-35 Kb	4-4.5 Kb	7-8 Kb	40-50 Kb	25 Kb	5 Kb
Titer (viral particle/mL)	10^{11}-10^{12}	10^{11}-10^{12}	10^{7}-10^{8}	10^{6}-10^{7}	10^{7}-10^{8}	10^{8}-10^{9}
Intracellular localization	Episomal	Episomal or genome integrated (in presence of *rep* gen in chromosome 19 of host's genome)	Genome integrated	Episomal	Episomal	Episomal
Expression	Transient	Transient or stably	Stably	Transient	Transient	Transient
Advantages	- Capacity of infection to diving and no-dividing cells - High titers - Very efficient gene transfer	- Naturally replication-deficient - Possibility of site-directed integration - Can enter in a latent infection resulting in long-term transgene expression	- Infection efficiencies close to 100% - Stably expression after integration - Easy to manipulate	- High capacity for bearing foreign DNA - Broad host range - Can infect neurons	- Broad host range - Safety - Strong expression levels	- Capacity of infection to diving and no-dividing cells - Non pathogenic - High recombinant protein concentration
Disadvantages	- Possibility of RCP* generation	- Low transgene capacity	- Only infection of dividing cells	- Possibility of RCP* generation	- Pre-existing immunity	- Low transgene capacity

- Some groups are oncogenic	- Possibility of RCP* generation	(excpetion: lentivirus)	- Possibility of RCP* generation

From the previous summarized viral vectors, adenovirus was the final choice in this present work. Adenovirus (AdV) vectors were initially developed for gene therapy vector production. Their capacity to infect both dividing and non-dividing cells, together with their relative ease for preparation and purification, made them one of the preferred vectors for gene therapy purpouses[27]. However, AdV vectors induce potent innate and adaptive immune responses, resulting in the rapid elimination of transduced cells due to generation of T and B cells against AdV and transgene product antigens. Hence, their usefulness for gene replacement was limited by their high immunogenicity[22]. Their aptitude for immune response inducement, have resulted in their extensive study as vaccine carriers over the last 20 years[28]. Moreover, AdV can be used as expression vector for production of recombinant proteins. Among their main advantages, the following should be pointed out[29]:

1. The high titres that can be achieved after infection (10^{11}-10^{12} virus particles/mL).
2. The great efficiency of gene transfer, almost about 100%.
3. The broad cellular host range that they can infect, and thus in which they can be produced). Namely: HEK293[30], 911[31], PTG6559[32], PER.C6[32], GH329 , N52.E6[33], HELA-E1[34], UR[35] and VLI-293.
4. The large sequences of foreign DNA that they can accommodate. Theoretically up to 30kb can be inserted in AdV genome, although a maximum of 8kb has been achieved in the currently used adenovirus vectors.
5. The ease of *in vitro* manipulation.
6. They do not integrate into de host chromosome, so they do not inactivate genes or activate oncogenes.

Taking into account all the adenovirus adantageous features, this expression system was selected as one of the study cases for the development of a bioprocess for protein production.

1.2.2.2. GENERATION OF STABLE CELL LINES FOR PROTEIN PRODUCTION.

Stable long-term expression of a gene of interest can be either achieved by eukaryotic vectors that harbour elements for episomal maintenance in the nucleus of a transfected cell, or via direct integration of the transfected plasmid into the target cells genome. Episomal vectors present some attractive features such as avoiding host gene disruption, insertional mutagenesis, and the

possibility of transgene silencing if the insertion occurs in condensed heterochromatin. However, episomal stability is often limited, resulting in gradual loss of transfected vectors that can only be prevented by sustained antibiotic selection eliminating cells that have lost the plasmid. Furthermore, the functionality of episomal plasmid elements is often restricted to certain species. Although integration into the host cell chromosome is a rare event and for most purposes clonal events have to be isolated, stability of the intended genetic modification is usually much higher. This is the main reason that makes integration of the gene of interest into host genome the most extended strategy for recombinant protein production in stable cell lines.

DNA can become integrated into chromosomes by two main processes: homology-dependent means and illegitimate integration[36]. Illegitimate integration is typically 1000–10.000 times more frequent than targeted integration (i.e. homology-dependent)[37]. However, as previously stated, the site of integration has a major effect on the transcription rate of the recombinant gene (a phenomenon known as the position effect)[38]. Aside from the consequences related to breaking down host's DNA or the integration into inactive heterochromatin it has also been reported transgene expression inactivation (also called gene silencing) even if the insertion occurs in euchromatin region. This is probably because of the influence of neighboring condensed chromatin.

On the other side, homologous recombination between transfected plasmid DNA and the genome rarely occurs[39,40]. One way to enhance the probability of targeted integration is to use enzymes, such as bacteriophage P1 Cre recombinase, lambda phage integrase or yeast Flp recombinase to exchange DNA between the genome and a transfected plasmid. These enzymes catalyze the exchange at high frequency if the donor and recipient DNAs are bordered by specific attachment regions (this methodology will be explained in more detail below)[41,42]. The identification of a highly active site of transcription before cloning the gene of interest, results in significative reduction of the screening for high-producer clones.

Two strategies for the obtention of stable cell lines producing the antigenic protein of the capsid of PCV2 (Porcine Circovirus type 2) will be explored in the present research project. The first one is based on the random integration of the gene into the hosts' genome after the transfection of a plasmid vector encoding for the gene of interest. The second one relies on the homologous recombination between donor vector and the host genome, in a targeted region previously identified as highly-active transcriptional region.

1.2.2.2.1. Ilegitimate transfection vectors and RMCE (Recombinase Mediated Cassette Exchange) for stable cell lines generation.

The non-specific or non site-directed cloning is the simplest strategy for animal cells transfections, in which the region of the genome where the foreign gene is inserted is *a priori* unknown. Consequently, the efficiency of the DNA transcription cannot be predicted, nor the recombinant protein production.

To improve the efficiency of stable cell line production with this strategy, a bicistronic mammalian expression vector has been used. Bicistronic vectors contain an internal ribosome entry site (IRES), which enables the coordinate co-expression of two genes with the same vector. Although translation initiation of eukaryotic mRNAs occurs almost exclusively at the 5' cap[43,44], the IRES allows ribosomes

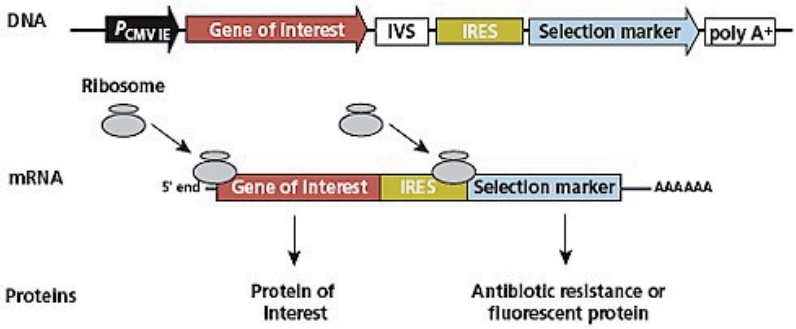

Figure 1.5. Scheme of the expression of genes encoded in a bicistronic vector.

to bind and initiate translation at a second, internal location. Thus, two proteins are expressed simultaneously from the same bicistronic mRNA transcript (Figure 1.5). The use of bicistronic vectors avoids the co-transfection of the vector expressing the protein of interest and the vector expressing the gene encoding for the clone selection feature, which is less efficient and normally results in poor clone obtention. Also, the expression of the two genes under the same promoter ensures the expression of the gene of interest when cells are cultivated in selection media. When the selection gene and the gene of interest are expressed in the same vector but under different promoters, the lost of the latter is usually observed, as it does not confer any advantage to the cell harbouring the plasmid.

The second strategy used to generat stable cell lines is the directed insertion of the gene of interest by Recombinase Mediated Cassette Exchange methodology (RMCE). This technology allows targeted integration of transgenes precisely into defined expression hot spots of the host cell genome[45,46]. RMCE can be best described as a two-step molecular “cut and paste” mechanism that relies on site-specific recombination carried out by a group of proteins called recombinases[47]. Recent distinguished reviews have been published revising molecular basis and advances in this technology [48–51] and here we will only focus on the application of this technology to generate stable cell lines. In

the first step, a reporter gene cassette is randomly integrated into a host cell line of interest (Figure 1.6). The expression of the reporter gene serves as a marker that permits screening for the desired expression characteristics (e.g. high and stable expression). Once isolated and confirmed as unique integration site, those "tagged" clones function as master cell lines. With the help of DNA recombinases such as the Cre[52–54] the Flp recombinase[55–57] or the phiC31 integrase[58] the tagged genomic loci of the master cell lines can be recycled by the integration of any gene of interest. This second step is usually refered to as "targeting" the host cell. Because the recombinase-driven cassette exchange itself is a highly site-specific event, all positive expression characteristics of the master cell line are transferred to the resulting subclonal producers.

In the present work, the Flip/*FRT* (Flipase/Flipase Recognition Target) system has been applied (Table 1.4). The FRT sites flanking the reporter gene (i.e. the wild type and a synthetic variant) cannot recombine with each other. This enables to overcome the major drawback of RMCE technology, which is the excission of the cassete by recombination of FRT sites. The specific reporter gene was the Green fluorescent protein (GFP), which enables the relative quantification of the expression levels of the gene. This feature was used in combination with single copy assessment of the reporter gene in order to generate the tagged master cells that will be targeted with the gene of interest. Following this strategy the high level of fluorescence in a given cell can be related to the integration of the tagging vector in highly translated region of the chromatin of the host's genome (also called "hot spots"). This enables to generate cell lines with consistent expression of the gene and it reduces the time to select high-producers after RMCE up to only seven weeks[59].

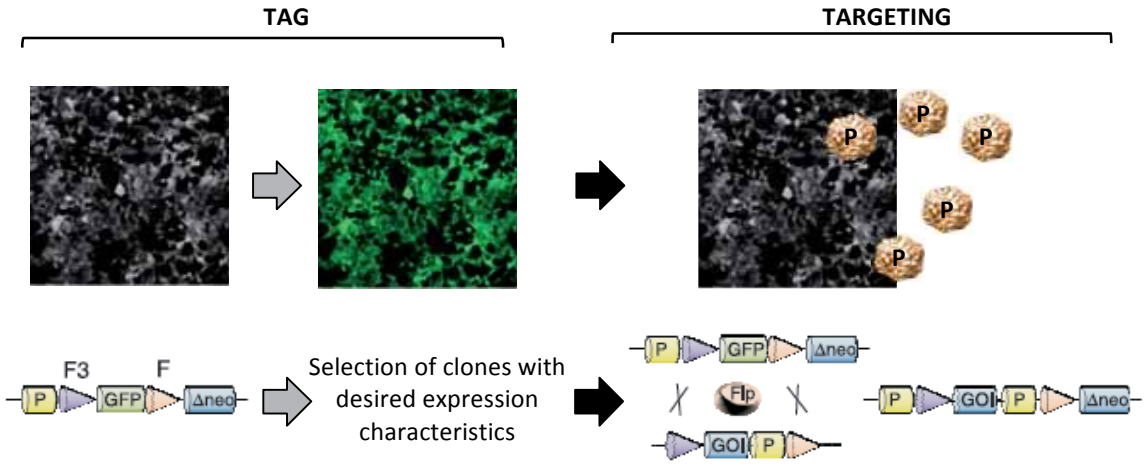

Figure 1.6. Scheme of Recombinanse Mediated Cassette Exchange (RMCE) methodology. Adpadted from[59]

Table 1.4. General and specific (i.e. applied in this work) characteristics of key parameters of RCME.

	Tagging method	Tagging gene	Recombination system
General	Random integration: - Reagent-based methods (lipids, Ca2PO4, cationic polymers…) - Instrument-based methods (electroporation, microinjection…) Viral vector infection (retroviruses, AAVs…)	Antibiotic resistance Bioluminiscence Chemiluminiiscence SEAP u-PA_{LMW}	Cre/loxP Flp/FRT Φ-C31/attB-attP Φ-BT1/attB-attP
Specific case	Electroporation Lentiviral infection	GFP	Flp/FRT

Abbr: SEAP (human secreted alkaline phosphatase, u-PA_{LMW} (low molecular weight urokinase-type plasminogen activator)

1.2.3. BIOPROCESS ENGINEERING

Bioprocess engineering is a wide field of science, which deals with the design and development of all processes involved in the manufacturing of products from biological material. It conglomerates knowledgment and research from a great spectrum of science including biology, data analysis, computing and chemical engineering. It can be applied to any part of the bioprocess itself, ranging from media definition, to cultivation parameters, culture strategies and downstream processes. In this work, we have focused on optimizing processes taking place into the bioreactor, by means of cell culture medium improvement and cell culture strategies development.

The cell media is a key element of all bioprocesses. Media not only have to contain all the nutrients and metabolites needed for cell growth and protein expression, but also have to provide with appropriate environmental conditions for cell expansion. Culture media contain a mixture of amino acids, glucose or other carbon sources, salts, vitamins, growth factors and other nutrients. Althogth the big efforts done by the media manufcaturers in order to design an universal media for a myriad of cell lines, the nutritional requirements vary substantially among cell lines, and these differences are partly responsible for the extensive number of current medium formulations.

Initially, the growth factors used to supplement media were often derived from the serum of animal blood, basically fetal bovine serum (FBS) or bovine calf serum (FCS). However, the regulatory agencies have baned this components in media for medical applications of biotechnological products because of the potential risk of contamination with virus, prions or other small molecules. For this

reason media manufacturers have developed an extense list of animal derived component free (ADCF) and also chemically defined media. A proper media selection is a very important issue in terms of increasing the bioprocess productivity and therefore, bioprocess profitability. Once the cell medium is selected, the study and development of culture strategies is a good approach in order to pursue in bioprocess optimization towards high cell density cultures.

Most of the established processes of cell culture are based on batch systems, which offer the advantage of simplicity and allow to culture cells in suspension in homogeneous bioreactors. In these culture systems cells reach a maximum concentration quite poor and, when this cell concentration is reached, their growth and viability sharply decay. There are different reasons for this decay but the most usual are nutrient exhaustion (mainly glucose and glutamine in batch cultures) and toxic metabolite accumulation, among which lactate and ammonium have received much attention in the past[12]. All these factors, together with the intrinsic low growth rate of these types of cells, render low productivities in these processes that are compensated, in many cases, by the high added value of the obtained products. The production capacity needs arising as the numerous new drugs under development based on mammalian cell culture impose engineering challenges for the intensification of the culture systems to increase productivities and minimize reactor volumes[60].

The media commonly used in batch cell cultures contain high concentrations of nutrients needed for cell growth, being glucose and glutamine the quantitatively main components and the main energy and carbon source (glucose) and nitrogen source (glutamine). However, these high concentrations of nutrients cause a metabolic deregulation in the inflows of these substrates. The deregulation in the cellular metabolism involves a rapid consumption of glucose and glutamine, resulting in their fast depletion and thus the end of the culture.

To overcome these disadvantages, other alternative cell culture strategies providing an environment closer to the physiological state of cells in in vivo are available, such as media fortification, fed batch or perfusion processes. However, to effectively implement all these culture system it is necessary to have measures (preferable on-line or in-line) that allow the continuous knowledge of concentration and activity of cells and the concentration of key compounds, especially those nutrients that are needed to be maintained at certain levels.

The simplest culture strategy that can be implemented is the fortified bacth, which consists in increasing the nutrients concentration of the initial cell medium. This strategy icrease the availability

of the nutrients, but still the limitations of by-products accumulation described in batch processes remains. Another possible strategy is based in controlling the concentration of the nutrients in the culture broth. Fed-batch systems allow increasing the concentration of viable cells in bioreactors, given that a more balanced and rational nutrient feeding allows controlling nutrient exhaustion[61,62]. Perfusion cultures systems offer a possibility for process intensification, although are observed often as complex strategies. They combine two important concepts: continuous culture and cell retention. Different perfusion systems have been developed[63–65] because of their production advantages in terms of higher productivity and, therefore, lowering production costs.

In this work a preliminary study of strategy cultures is tackled in shake platform, based on nutritional supplements screening, and manually controlled addition strategies. This preliminary study will be the base of fully controlled feeding strategies in bioreactor, what is a ongoing work within the group.

1.2.4. METABOLIC ENGINEERING

Metabolic engineering is generally referred to as the targeted and purposeful alteration of metabolic pathways found in an organism in order to better understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly[66]. In essence, metabolic engineering is the application of engineering principles of design and analysis to the metabolic pathways in order to achieve a particular goal. Early strategies to reach these goals seem more of an art with experimentation by trial-and-error. However, in parallel to the milestone achieved in the 1980s by Cohen&Boyer[67] when they first successfully introduced foreign genes into a bacteria cell, a switch from this artistic approach to a more systematic and rational approach also occurred. This led to the publication of the first papers enumerating all possible routes connecting a substrate with a target product, the thermodynamic analysis of such pathways, investigations on the distribution of kinetic control, and the design of genetic circuits to bring about a desired pattern of gene expression and product synthesis, among others. These activities culminated in two seminal papers[68,69] that essentially initiated the field of metabolic engineering. This scientific approach would involve the use of recombinant DNA technology and a better understanding of cellular physiology to modify intermediary metabolism. It must be pointed out that metabolic engineering was differentiated from genetic engineering by its distinct focus. Whereas the former field was focused on investigating the properties of integrated metabolic pathways and genetic regulatory networks, the latter was more directed to the study and manipulation of individual genes and enzymes. Metabolic engineering must be also differntiated from other overlapping fields such as synthetic biology. Synthetic biology relies on the building of functional pathways by switching, overexpressing

or deleting genes to produce a few milligrams of a product. However, metabolic engineering has a distinct industrial dimension, because it aims to construct microbes that can carry out cost-effective production of a desired product[70].

Metabolic engineering has been applied to improve the yield and productivity of native products synthesized by microorganisms[71–73]. Another application is to extend the range of substrates[74,75], and a third class of applications is the production of products that are new to the host cell[76,77], or entirely novel (e.g. biopolymers[78,79]). The improvement of general cellular properties, such as the ability to withstand hypoxic fermentation conditions[80], alteration of nitrogen assimilation pathway[81], or prevention of overflow metabolism[82] is another class of applications of metabolic engineering. There are many environmental applications as well[83], and yet another area where pathway manipulation can be very profitably applied is in the manufacturing of chiral compounds as intermediates in the synthesis of pharmaceutical products. Finally, another broad area of metabolic engineering applications is in the medical field for the analysis of the metabolism of whole organs and tissues as well as the identification of targets for disease control by gene therapy or nutritional strategies[84]. Metabolic engineering comprises relevant subjects and excellent reviews covering them are indicated in the Figure 1.7. In this Introduction we will focus on metabolic balance analysis and flux balance analysis.

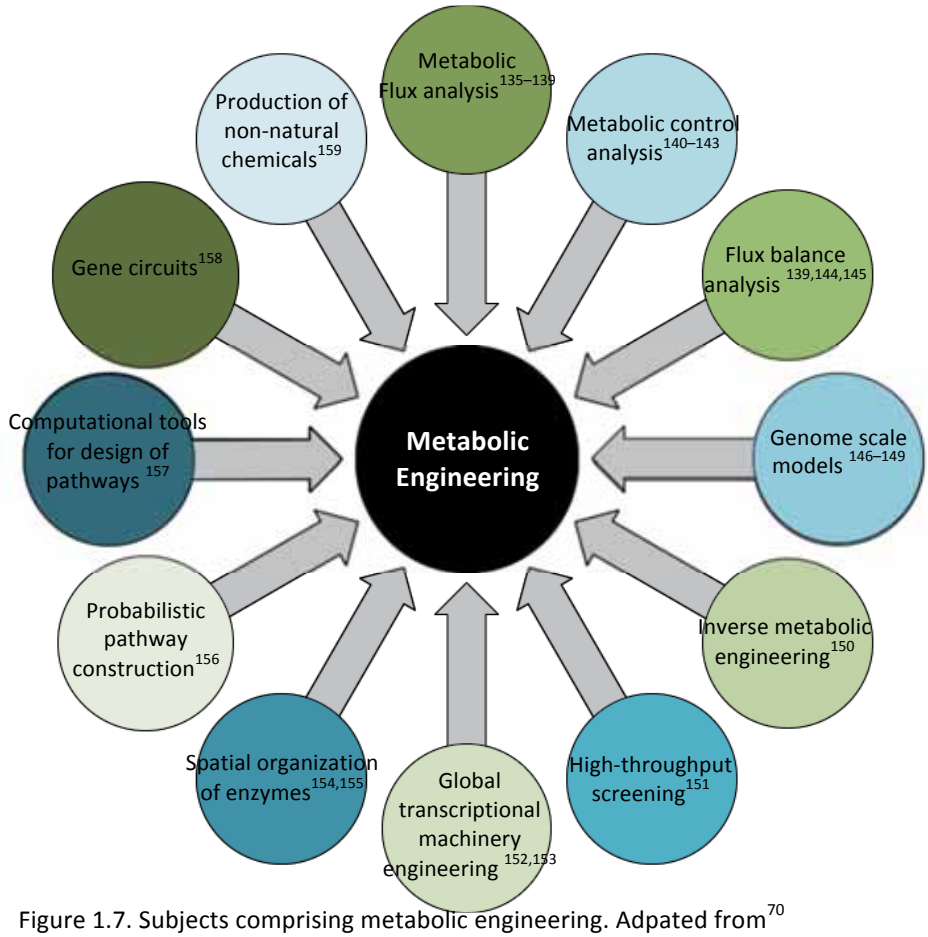

Figure 1.7. Subjects comprising metabolic engineering. Adpated from[70]

In metabolic engineering, flux analysis plays an important role for the generation of new biological knowledge of the celular system, system-wide analysis of celular phisiology, and in developing metabolic engineering strategies at the systems-level. Up to date, various methods have been developed and they can be classified as shown in Figure 1.8.

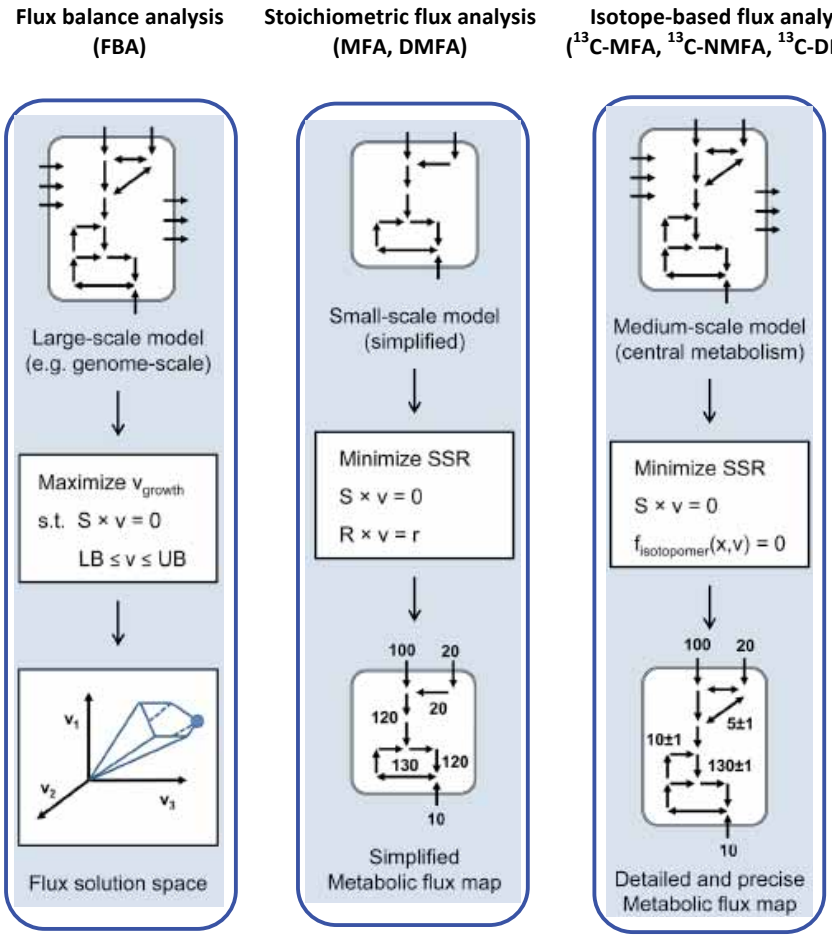

Figure 1.8. Schematically representation of the three groups in which flux anlysis can be classified. Adpadted from[85].

Metabolic flux analysis (MFA) relies on balancing fluxes around intracelular metabolites within an assumed metabolic network model. MFA uses extracelular measured metabolic rates and assumes pseudostationary state (PSS) to estimate the metabolic fluxes. The main advantage is that is easy to apply and thus, accesible to many researchers[85]. The major drawback is that in order to be able determine meaningful flux distributions, the biochemical network model is usually simplified (e.g. by leaving out reactions that are considered insignificant, or unobservable) to such an extent that a handful of measured extracellular rates can provide sufficient data to determine the remaining fluxes in the mode. As a strategy for disminishing this drawback, labelling of isotopomers (^{13}C) in order to measure measure intracelular meatbolic fluxes can be applied. Alternatively, flux balance

analyses are used to quantify fluxes in undetermined systems. In this methodology, in addition to applying constraint for measured extracelular rates, inequality constraints such as upper and lower bounds on fluxes are used, and an biological objective is imposed on the model, for example, maximum growth rate or máximum ATP production. Hence, FBA uses optimisation-based simulation techniques to analyze celular metabolism under a specified environmental or genetic condition. Because of their properties, MFA ^{13}C-MFA approach are used primarily for measuring in vivo metabolic fluxes, whereas FBA and other related in silico approaches are used largely for evaluating the biotechnological potential of organisms and identifying genetic manipulations that could improve product yields and cell-specific productivities.

Finally, the other flux analysis methodologies depicted in Figure XXX avoid the assumption of pseudostationary state and consider dynamism of labelled tracers (isotopic non-steady state, ^{13}C-NMFA) or of celular growth and intracelular metabolism dynamics (dynamic metabolic flux analysis, DMFA). Dynamic flux analysisi should be more convenient for the understanding and optimization of industrial batch and fed-batch bioprocesses in which steady state is not reached and cells are continuosly adapting to the environment[85]. The incorporation of labelling tracers to DMFA (13C-DMFA) has also recently been applied, but there is a clear need for further research and development in this área to make these methods more accesible and more easy to implement[85].

1.3. SELECTION OF MODEL RECOMBINANT PROTEIN FOR BIOPROCESS DEVELOPMENT. STUDY CASE: PORCINE CIRCOVIRUS SEROTYPE 2.

1.3.1. GENERAL INFORMATION

Porcine circovirus (PCV) is a small, non-enveloped virus with single-stranded circular DNA genome classified into the Circoviridae family[86]. Type 1 PCV (PCV1) was discovered in 1974 as a contaminant of the porcine kidney cell line PK-15[87]. Although PCV1 is present in swine population worldwide, it does not cause clinical disease in pigs. A variant strain of PCV, designated type 2 PCV (PCV2), was discovered in Canada in the mid-1990s and it is considered the primary causative agent of an economically significant collection of disease syndromes in pigs, currently known as porcine circovirus associated diseases (PCVADs) in the United States or porcine circovirus diseases (PCVDs) in Europe[88].

PCV1 and PCV2 have similar genomic organization with two major ambisense ORFs flanking the origin of replication (Figure 1.9)[89]. The largest (ORF1) codes for the replicase proteins, Rep and

Rep'[90]. ORF1 is oriented in the sense direction relative to the origin in the PCV2 genome. Rep is translated from the entire ORF1 transcript, whereas, Rep' is derived by alternative splicing of the ORF1 transcript[90–93]. Both proteins are essential for viral replication[90,92,94–97]. ORF2 encodes the viral capsid protein, which has the ability to bind to the host cell receptor and which contain the immunodominant antigenic epitopes[98–103]. Recently, two additional ORFs have been described and named ORF3 and ORF4. The former, encodes for a protein that its not related to viral replication but it has been reported to cause apoptosis in virus-infected cells and to play an important role in the pathogenesis of PCV2 infection[104,105]. ORF4 is neither essential for PCV2 replication but it has been related o caspase activity suppression and regulation of T lymphocytes during PCV2 infection [106].

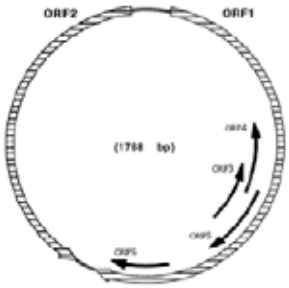

Figure 1.9. Scheme of PCV2 genome. Adapted from[107].

1.3.2. PCV2 STRAINS AND GENETICALLY VARIATON

Sequencing of the PCV2 genome has shown several different genotypes. Up till now 5 genotypes have been described and letter-named, based on a proposal form an EU PCVD-consortium resulting in genotypes a, b, c, d and e[108]. Type a and b seem to have a worldwide distribution whereas type c, d and e have only been found in Denmark, China and Thailand, respectively[109]. It is estimated that the rate of nucleotide substitution for PCV2 is on the order of 1.2×10^{-3} substitutions/site/year, the highest yet recorded for a single stranded DNA virus. This high rate of evolution may allow PCV2 to maintain high evolutionary, therefore facilitating the rapid emergence of PCV2 worldwide.

1.3.3. PCV2 INFECTION IS AN OPEN DOOR FOR OTHER DESEASES DEVELOPMENT.

PCV2 infection is characterised by a high prevalence of infection but a low morbidity, and thus not all animals infected with PCV2 will develop clinical signs of PCVDs. Therefore, it has been reported that the infection with PCV2 is necessary but no sufficient to develop PCVDs. Other factors (usually called triggering factors) play an essential role on the outcome of the infection. Among the triggering factors, the main ones would be the following:

1. **Swine pathogen co-infections**: Coinfection with other viral and bacterial pathogens has been proved to cause an increasing incidence and a more severe clinical course of disease. The agent that generates the greatest risk is porcine reproductive and respiratory sindrome virus (PRRSV). Other agents include porcine parvovirus (PPV), Mycoplasma hyopneumoniae, and TTV, which is not associated with the disease on its own but is present in many pig populations[110].

2. **PCV2 specific strain**: some differences on the virulence of the infection related to differences on the genomic sequence (hence, different strains of te virus) have been reported[111].

3. **Sow effect:** Calsamiglia et al[112] reported that low PCV2 antibody titres in sows were positively related with pig mortality. This fact may imply that PCV2 maternally derived antibodies might protect against piglet mortality. This suggestion was previously observed under field and experimental conditions[113,114].

4. **PCV2 infection timing:** A longitudinal study in seven PMWS farms showed an increasing risk of PMWS if piglets were infected early (before 7 weeks of age), whereas reduced risk was found if piglets were weaned after 21 days[115].

5. **Management practices**: Environmental factors such as drafts, overcrowding, poor air quality, mixture of age groups, and other ''stressors'' may exacerbate the severity of the disease[116].

6. **Host Factors**: All breeds of pigs appear to be susceptible to infection, and clinical disease has been observed in many purebred and crossbred pigs. However, variations in the onset of the adaptive immunity after PCV2 infection have been reported and this may account for varaition in PCV2 replication in pigs[117]. The levels of passively acquired antibodies might also play a role on the immunogenic host response to infection.

1.3.4. DISEASES AND SYNDROMES ASSOCIATED TO PCV2 INFECTION.

As it has been previously stated, PCV2 is the infectious agent that causes different síndromes, which are grouped under the the umbrella term porcine circovirus disease (PCVD), which includes wasting, increased mortality, respiratory signs, enteritis, reproductive failure, and porcine dermatitis and nephropathy syndrome (PDNS)[118]. In Europe, the term porcine circovirus disease (PCVD) is more frequently used in the literature. The most significant manifestation of PCVDs is the post weaning

multisystemic sindrome (PMWS); accordingly, most research efforts have been directed towards this area. This disease affects pigs between 7 and 16 weeks old in the United States and 5–12 weeks old in Europe. This age difference is most likely related to variations in management practices and vaccination timing between producers in the United States and Europe. Mortality is usually around 10% (range 4–20%), but can reach up to 50%. Since the clinical course of wasting can be long (therefore, decreasing economic efficiency) 70–80% of pigs that develop PCVD are euthanized[107].

1.3.5. WHY PCV2 AS A TARGET?

In the last 30 years, several new swine diseases have been described. Two of the most global and economically important ones are the porcine reproductive and respiratory syndrome (PRRS) and the post-weaning multisystemic wasting syndrome (PMWS)[119]. PCV2 infection is present in every major swine-producing country in the world and the number of cases of PCVD is rapidly increasing. Different statistics have been reported depending on the geographic area, mainly covering United States, Europe and Asia. Some data is summarized below in order to get an idea of the current state of PCVDs:

- In Part II of the Reference of Swine Health and Health Management in the United States published in the year 2000 by the U.S. Department of Agriculture (USDA), PCVD prevalence was determined by United States Department of Agriculture. The orevalence was estimated at nearly 94% of the U.S. pig inventory[110,120]. Other studies have reported re-emerging of PMWS during last two years in North and South-America, being considered the most important disease in the pig population in many of their countries[95,121]. In Europe an Asia, the prevalence and severity of PMWS outbreaks has diminished to some extent during the last years. However, the disease is still significant or very significant in some countries of those geographical areas and there are several countries (i.e. UK, Denmark, Austria, Ireland and Sweden) that consider PMWS as a major disease in its national swine production[122].

- Since 2003, there has been a drastic global shift in the predominant prevalence of PCV2b in commercial swine populations, as well as an increased severity of clinical PCVD[95,121,123–127]. The current PCV2a-based vaccines have been shown to confer cross-protective immunity against PCV2b[128–131], but the antigenic profiles of PCV2a and PCV2b are not identical, and it remains to be seen whether new vaccines based on the PCV2b genotype could provide superior protective immunity against PCV2b field strains compared to the currently available PCV2a-based vaccines [88].

- The disease has major economic, public health, and animal welfare impact on the pig meat industry worldwide. The economic cost of PMWS across the EU is estimated at €562M to €900M. Factors underlying the economic losses include fewer pigs at slaughter, reduced feed conversion rates, increased costs for management and medication of sick pigs, and costs of secondary diseases following PMWS-associated immunosuppression[132].

- Vaccination against PCV2 has only been recently introduced in cattle industry. However, some studies have already emphasized the benefits from this vaccination. Even in farms with no reported mortality due to PCV2 infection, positive economic impact has been reported after vaccination (Table 1.5). This can be explained by the severity of the damage caused to the pigs by the PCV2 viraemia in the pig's body and the damage it does internally. The pig has to fight the infection and uses up energy to do so, reducing liveweight gain. This fact has major economic impact; for example, in the US low weight (wasted) pigs are heavily penalised, fetching under half the price/kg than normal pigs[133].

Table 1.5. Cost/benefit for vaccination piglet with PCV2 piglet vaccines. Adapted from[134].

	Uninfected	Infected				
Mortality (%)	0	0	1	3	5	7
Lost liveweight/pig (kg)	0	2.5	3.0	4.0	5.0	6.0
Batch mortality (%)	100	100	99	97	95	93
Total batch value (€1.10/kg)	11,000	10,725	10,563	10,243	9,928	9,616
Income lost rate (%)	0	2.56	4.14	7.39	10.81	17

In a nutshell, PCVD is an emerging disease with major implications in the pig industry worlwide and PCVD vaccination is a relative new market whith an increasing growth projection. Investigation in this field may provide with new solutions that give answer a proven necessity in the farming business.

Since there are already some commercial available vaccines against the disease that have proven their efectiveness (Table 1.6), the main objective of this research project will be to find ways of increasing the product competitivity by optimizing the bioprocess used to obtain the vaccine and therefore reducing its final cost. The existing vaccines currently available will be used as reference to compare the immune response generated by pur vaccine candidate, therefore stablishing if the new production methods will produce a solution equivalent in eficcacy to those already in the market.

Table 1.6. Current available vaccines against PCV2. Adpated from[88].

Vaccine	Manufacter	Antigen	Usage
Circovac®	Merial	Inactivated PCV2a virus	Breeding sows/piglets (>3weeks old)
Ingelvac CircoFLEX®	Boehringher Ingelheim	PCV2a capsid protein	Piglets (>3weeks old)
Circumvent®	Intervet (Merck)	PCV2a capsid protein	Piglets (>3weeks old)
Porcilis® PCV	Schering-Plough (Merck)	PCV2a capsid protein	Piglets (>3weeks old)
Fostera™ PCV*	Pfizer	Inactivated attenuated chimeric PCV1-2a virus	Piglets (>3weeks old)

*(formerly Suvaxyn® PCV2 One Dose™)

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CHAPTER 2: OBJECTIVES

This work has been developed into the Cellular and Biotechnology Engineer Group, which main objective is the development, optimization and scaling up of bioprocesses based on cell culture with a wide range of cells, specifically with mammalian cells. In particular, this work is focused on the development of an efficient and scalable bioprocess for recombinant protein production in HEK293 cell line. As a model of heterologous protein production, the capsid of the Porcine circovirus serotype 2 (PCV2) will be produced.

This main objective can be divided in the following sub-objectives:

- The study of cell media and cell culture strategies towards the maximisation of HEK293 viable cell density.
- The generation of recombinant Adenoviral vectors encoding for the gene of interest and the study of the best infection parameters in order to maximise adenoviral vector production and recombinant protein production.
- The generation of stable cell lines producing the model protein selected (r-CapPCV2).
- The study of HEK293 cell line physiology and metabolism and the implementation of metabolic tools for cell line and cell culture optimisation through metabolic engineering.

CHAPTER 3. RESULTS (I): HEK293 CELLS IN VITRO CELL CULTURE

3.1. INTRODUCTION

As it has been mentioned in the introduction of this work, to achieve the full potential of a given cell line it is important to create a suitable culture environment that provides the optimal chemical and physical conditions for cells to grow and produce the protein(s) of interest[1]. Also, understanding cell culture processes is a key factor in order to sustain cell viability in high-density cultures. On this direction the study of cell media and cell culture strategies must be addressed on the initial stages of a bioprocess development. Since culture is strongly related to cell growth, product yield and even to product quality, culture medium changes should be avoided at late phases of the bioprocess progress. Additionally, for feasibility, economical and time consumption concerns it is highly recommended to explore the potential of diverse bioprocess approaches at lab scale, although some modifications could be mandatory on the scaling-up process. As stated in the Introduction, in the present project several strategies for the production of the protein CapPCV2 (r-CapPCV2) are explored. Although they are quite different in terms of how the protein is produced, all the processes have in common the biocatalyst used for obtaining the protein. These biocatalysts are HEK293 cells.

The optimisation of a bioprocess should be technically and economically addressed. From the technical perspective the researcher is in pursuit of the maximal increment of volumetric productivity (V_p). V_p is directly proportional to the biocatalyst concentration and to the biocatalyst specific production rate of the product of interest (Eq.3.1). From the economical point of view the main objective is to achieve the reduction of the bioprocess costs as much as possible. A good balance between the two viewpoints would lead to an optimized bioprocess. In this first chapter, the increment of the biocatalyst concentration will be addressed as a first step of the bioprocess optimization.

$$\boldsymbol{V_p}\left[\frac{\boldsymbol{mass\ product}}{\boldsymbol{volume}\times\boldsymbol{time}}\right] = \boldsymbol{X_v}\left[\frac{\boldsymbol{biocatalyst}}{\boldsymbol{volume}}\right]\cdot\boldsymbol{q_p}\left[\frac{\boldsymbol{mass\ product}}{\boldsymbol{biocatalyst}\times\boldsymbol{time}}\right] \quad \text{Eq. 3.1}$$

3.2. CELL MEDIA SELECTION

3.2.1. CELL MEDIA SCREENING. MEDIA SUPPLEMENTATION WITH FBS AND ADCF SUPPLEMENT (CB5).

Historically, simple chemical defined media were used for mammalian cell culture (i.e. MEM, DMEM, RPMI, Ham's F-12). Access to the formulation of these media is unrestricted which is an advantage for performing a wide range of in vitro studies. Therefore, these media are still widely used in basic research cell-based studies, but they have been slowly replaced by other media for industrial purposes. Over the last decade, the increasing demand on biomolecules and the emerging market of biosimilars[2] has pushed industries to increase efforts on development of cell media to achieve high cell density cultures and thus increasing volumetric productivity of a given process. Also, the paradigm of one-media-fits-all approach has been changing towards the development of complex formulations optimized for a particular cell line and even for a particular application or bioprocess[3]. Altogether has result in a myriad of commercially available culture media and cell media supplements.

It is also important to mention that the recent introduction of regulations requiring the removal of serum (FBS, fetal bovine serum or FCS, fetal calf serum) and other animal-derived components in all biomanufacturing processes has led to the introduction of leaner, more defined animal-derived-component-free media (ADCF)[3]. However, the substitution of serum in many of the formulations has been reported to cause a reduction of cell growth and productivity compared to those achieved with serum supplementation[4]. Therefore, a large number of industrial processes for recombinant protein production are currently performed with serum-supplemented media[5,6].

In the present project, three different ADCF chemically defined cell media were selected for screening on basis of previous work performed by the research group. Moreover, they were supplemented either with FBS or with ADCF nutritional supplement in order to compare its effect on cell growth rate and maximal viable cell density. Panel A on Figure 3.1 shows viable cell density (Xv) and viability profiles for the selected media. Cultures performed in HyQ SFM4HEK293 and HyQ SFMTransFx-293 showed better cell growth than HyQ CDM4HEK293, reaching maximum cell densities (Xv_{max}) around $3.5x10^6$ cell/mL, $2x10^6$ cell/mL and less than $1x10^6$ cell/mL respectively. Aside from cell concentration, the main difference between tested media was that viability was sustained longer over 85% when HyQ SFM4HEK293 medium was used.

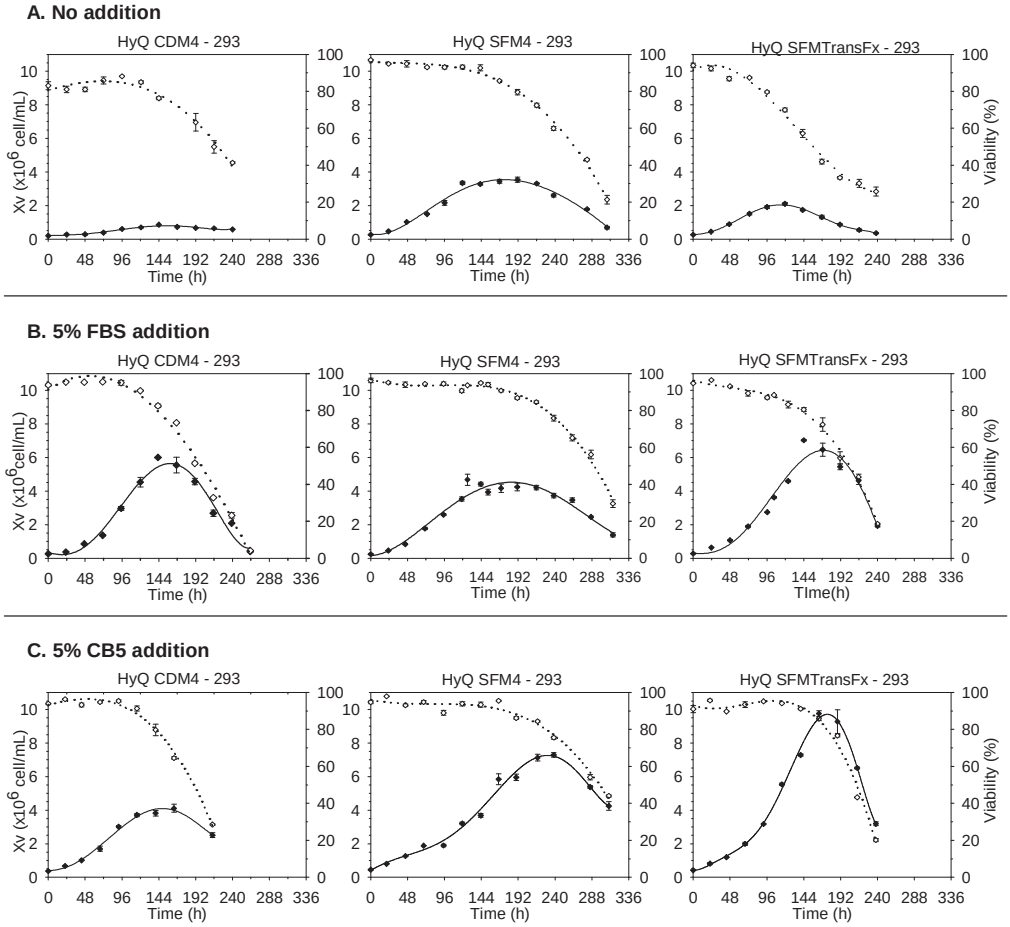


Figure 3.1. Viable cell density (Xv) (solid symbols) and viability profiles (empty symbols) of HEK293 cell cultures. Panel A: basal commercial media; panel B: commercial media supplemented with 5% (v/v) of FBS; panel C: commercial media supplemented with 5% (v/v) of CB5. On the three panels, from left to right: HyQ CDM4HEK293, HyQ SFM4HEK293 and HyQ SFMTransFx-293. The average from two cell count of three biological replicates are shown. Error bars represent the standard error of the replicates.

Maximum specific growth rates (μ_{max}) were estimated and compiled in Table 3.1. The μ_{max} value obtained for media HyQ SFM4HEK293 and HyQ SFMTransFx-293 was about 0.024 h^{-1} (which corresponds to a doubling time of 29 hours) as it was expected[7], and it was sustained for approximately 74 hours after inoculation (the exponential growth phase is expressed here as $t_{\mu max}$). However, μ_{max} value for HyQ CDM4HEK293 medium was only about the half (0.011 h^{-1}) meaning that the media is not adequated for the HEK293 strain used in this work.

Table 3.1. Kinetic parameters for HEK293 cultures corresponding to the profiles depicted in Figure 3.1.

		HyQ CDM4HEK293	HyQ SFM4HEK293	HyQ SFMTransFx-293
Basal medium	Xv_{max} (x10^6cell/mL)	0.85±0.0	3.53±0.21	2.10±0.12
	μ_{max} (x10^{-2} h^{-1})	1.06±0.01	2.46±0.14	2.43±0.03
	$t_{\mu max}$ (h)	96	74	74
5%FBS	Xv_{max} (x10^6cell/mL)	6.00±0.0	4.67±0.48	7.02±0.06
	μ_{max} (x10^{-2} h^{-1})	2.61±0.04	2.80±0.05	2.67±0.01
	t_{μ} (h)	95	71	71
5%CB5	Xv_{max} (x10^6cell/mL)	4.11±0.33	7.29±0.18	9.75±0.25
	μ_{max} (x10^{-2} h^{-1})	2.10±0.06	2.06±0.03	2.17±0.01
	t_{μ} (h)	92	69	116

Since all media selected are described as serum free media, the addition of FBS was assessed in order to evaluate whether the substitution of critical serum components have satisfactorily been performed without affecting cell growth. Once the FBS lot was selected (data not shown), the effect of its addition (5% v/v) to cell media was studied. The concentration of FBS was slected as a starting intermediate point based on previous work with mammalian cell lines[8] As it is shown on Panel B of Figure 3.1, FBS addition significantly increased maximal cell concentration in two cases: 7-fold increment for HyQ CDM4HEK293 (6±0.00 x10^6 cell/mL) and 3-fold increment for HyQ SFMTransFx-293 (7.02±0.13 x10^6 cell/mL). In HyQ CDM4HEK293 cultures was also remarkable the positive effect of FBS on μ_{max}. This value was more than doubled (from 0.011h^{-1} to 0.026h^{-1}) becoming comparable to the other cell media (values compiled in Table 3.1). On the contrary, the addition of 5%(v/v) FBS had unremarkable effect on HyQ SFM4HEK293 cell cultures in terms of μ_{max} (0.028 h^{-1}), Xv_{max} (4.67±0.82 x10^6 cell/mL) or viability profile.

In parallel to FBS addition experiments, supplementation of media with ADCF nutritional supplement recommended for HEK293 cell culture was performed in order to define a cell culture platform for those applications in which absence of serum is preferable. Cell Boost 5 was selected for the initial

screening due to manufacture's recommendation. The addition of 5% (v/v) of Cell Boost 5 solution (onwards CB5) enhanced significantly cell growth (Panel C Figure 3.1). In particular, this addition resulted into a 4-fold increment of Xv_{max} for HyQ CDM4HEK293 (4.11±0.58 $x10^6$ cell/mL), 2-fold increment for HyQ SFM4HEK293 (7.29±0.32 $x10^6$ cell/mL) and 5-fold increment for HyQ SFMTransFx-293 (9.75±0.44 $x10^6$ cell/mL).

Focusing on the calculated μ_{max} and $t_{\mu max}$ values (compiled in Table 3.1), it can be noticed that specific growth rate was higher when FBS was used as supplement whereas CB5 did not have a positive effect on this parameter, independently from cell media. Nevertheless, CB5 addition extended the exponential growth phase for 44h when HyQ SFMTransFx-293 was used, achieving higher Xv_{max} with 5% (v/v) CB5 supplementation in comparison to HyQ SFMTransFx-293 with 5% (v/v) FBS. In the case of HyQ SFM4HEK293 cell medium, $t_{\mu max}$ values were comparable for the three conditions tested. However, only a slight decrease on μ_{max} was detected beyond $t_{\mu max}$ (around 70h) when CB5 was used as supplement whereas a dramatic drop of this value was noticed beyond $t_{\mu max}$ for non-supplementation or FBS addition, consequently affecting the final cell concentration achieved with HyQ SFM4HEK293 5% (v/v) CB5.

After the evaluation of these results, SFMTransFx-293 was selected for the experiments onwards, as the maximal cell density was achieved with this media.

3.2.2. SUPPLEMENTS CONCENTRATION STUDY (FBS AND CB5) USING THE SELECTED MEDIA (HyQ SFMTransFx-293)

In order to tune the final concentration of each supplement with the aim of increasing viable cell density and lowering the medium cost, a range from 2.5 to 10% (v/v) for FBS and 2.5 to 20% (v/v) for CB5 were evaluated using HyQ SFMTransFx-293 media.

The results concerning FBS supplementation are presented in Figure 3.2 and Table 3.2. The maximal viable cell density reached was approximately 7x10^6 cell/mL and μ_{max} was in the range reported for HEK293 cell line (0.024–0.027h^{-1}) for all FBS concentrations tested. On one hand, no negative effects associated with toxicity were observed at any concentration assessed, but addition of higher concentrations than 5% did not contribute to improve cell growth. On the other hand, μ_{max} and Xv_{max} were slightly lower when the addition of FBS was reduced to 2.5% (v/v), meaning that probably this concentration might have limiting effects. Thus, a concentration of 5% of FBS was selected as the preferred concentration for HyQ SFMtransFx-293 medium. Finally, it is remarkable that when HyQ

SFMTransFx-293 was supplemented with FBS μ_{max} values were higher compared to basal media (independently from the concentration evaluated) confirming the positive effect of FBS on this parameter encountered already on the prior media study presented.

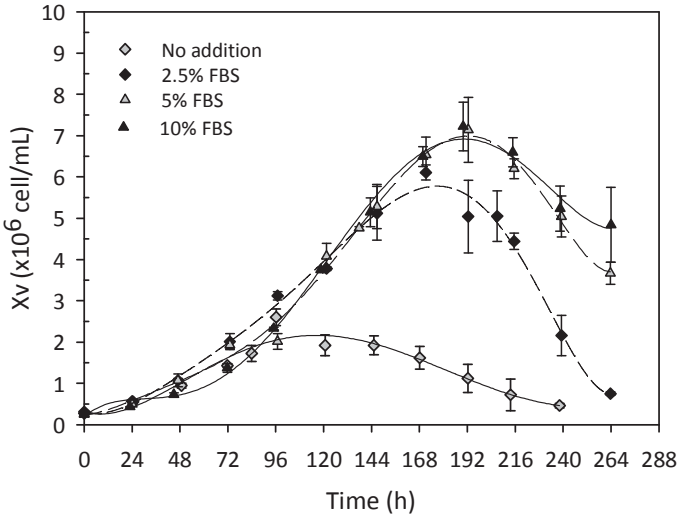


Figure 3.2. Growth profiles of HEK293 cell cultures in HyQ SFMTransFx-293 cell media supplemented with different FBS percentages in the range between 0 and 10% (v/v) final concentration. The average of two cell count of three biological replicates are shown. Error bars represent the standard error of the replicates.

Table 3.2. Kinetic parameters for HEK293 cultures corresponding to the profiles depicted in Figure 3.2.

%FBS (v/v)	Xv_{max} (x10^6 cell/mL)	μ_{max} (x10^{-2} h^{-1})	Viability to Xv_{max} (%)
0	2.60±0.20	2.27±0.67	86.24±0.69
2.5	6.11±0.18	2.60±0.31	84.21±4.48
5	7.1±0.79	2.67±0.20	83.05±1.99
10	7.22±0.59	2.43±0.13	76.05±0.39

Differently to what was observed in the FBS experiment, a proportional positive effect on Xv_{max} was noticed with the addition of CB5 up to a concentration of 10% (v/v) (Figure 3.3). At this concentration, μ_{max} was sustained for longer t_μ (144 h) in comparison to lower concentrations (116h), and consequently resulted in an increase on viable cell density reaching about 13x10^6 cell/mL. However, when cultures were supplemented with 20% (v/v) of CB5, no significant differences on μ_{max} were detected at the beginning of the culture, but this value decreased substantially beyond 96h, and thus, maximal cell density reached was limited to only 8x10^6 cell/mL. This fact might take place because by-products accumulation in a high-nutrient content media may increase osmolarity, affecting negatively to cell growth. Remarkably, CB5 addition did not significantly affect μ_{max}, but it indeed allowed the elongation of $t_{\mu max}$, resulting into a significant increment on the final cell density reached.

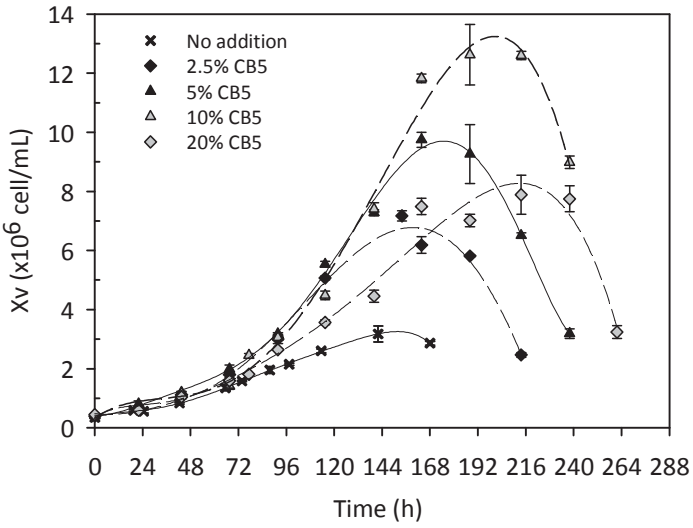


Figure 3.3. Growth profiles of HEK293 cell cultures in HyQSFMtransFx-293 cell media supplemented with different CB5 percentages in the range between 0 and 20% (v/v) final concentration. The average of two cell count of three biological replicates are shown. Error bars represent the standard error of the replicates.

Table 3.3. Kinetic parameters for HEK293 cultures corresponding to the profiles depicted in Figure 3.3.

%CB5 (v/v)	Xv_{max} ($x10^6$ cell/mL)	μ_{max} ($x10^{-2}$ h^{-1})	Viability to Xv_{max} (%)
0	3.18±0.28	2.03±0.23	84.01±0.27
2.5	7.17±0.17	2.19±0.10	91.23±0.49
5	9.75±0.25	2.17±0.04	92.06±0.20
10	12.63±1.02	2.08±0.05	92.23±0.53
20	7.89±1.14	1.94±0.10	91.61±0.85

The studies of cell media and nutritional supplementation presented so far enabled to highly overcome the maximal viable cell density reached when using the basal cell media. Specifically, a 6-fold increment on Xv_{max} was achieved in the best scenario (HyQ SFMTransFx-293 supplemented with CB5 at 10% (v/v) mainly due to the elongation of the exponential growth phase. Nevertheless, all the experiments hereto were performed in batch mode operation due to its simplicity for implementation. However, there are other cell culture strategies, which are known to be more efficient for achieving high cell density cultures (HCDC). In the following section of this chapter, the study of cell culture strategies will be addressed and evaluated in order to develop HCDC.

3.3. CELL CULTURE STRATEGIES INITIAL CHARACTERISATION IN SHAKE FLASKS.

There are four major cell culture strategies that are applied to mammalian cell-based bioprocesses:

Batch cell culture (Figure 3.4A) is the simplest strategy, where all the nutrients are added from the very beginning of cell culture[9]. This feature can lead to nutritional limitation due to nutrient toxicity or solubility concerns. Throughout cell culture evolution, media composition varies depending on the consumed nutrients and the by-products accumulation due to cell metabolism. As a consequence of these variations, cell growth is limited and once a certain cell density is reached, an irreversible death phase of cell culture occurs.

In **fed-batch** cell cultures (Figure 3.4B) a controlled addition of the required nutrients is performed along cell expansion. This progressive supplementation allows avoiding possible negative effects derived from substrate inhibition or nutrient depletion. Also, this strategy can be used to control the production rate of toxic by-products. Although fed-batch cultures permit to extend cell growth phase in comparison to batch cultures, at a certain point the accumulation of by-products or the increment of media osmolarity triggers cell death phase[9–11].

Continuous cell cultures (Figure 3.4C) are based on the feature that cells can be grown in a physiological steady state[9,12]. In a steady state, all culture parameters remain constant (i.e. culture volume, dissolved oxygen concentration, nutrient and product concentrations, pH, cell density) and thus, growth occurs at a constant rate. This is accomplished by the constant feeding of fresh media and the removal of exhausted media with cells at the same rate. As nutrient supplementation is controlled and there is a constant removal of toxic by-products, cell culture can be extended for months. Although being a strategy highly useful for cell culture study and cell physiology characterisation, it is not widely used in industrial bioprocesses since lower cell densities and product concentration are reached in comparison to discontinuous systems.

In **perfusion** cell cultures (Figure 3.4D) a continuous media feeding and exhausted media removal are also performed, but differently from continuous cultures, cells are retained into the bioreactor[9,13,14]. In comparison to batch and fed-batch, the perfusion mode allows prolonging cell expansion reaching HCDC, as well as it reduces the residence time of the product in the bioreactor. Besides, the perfusion strategy allows working at higher dilution rates and consequently greater cell densities and higher volumetric productivities are achieved in regard other culture strategies. Nevertheless, perfusion processes have also drawbacks: they are more challenging in terms of technical complexity and sterility assurance. Besides, large harvest volumes are continuously

accumulated, large fresh medium volumes are required, and there is the neeed to generate various product batches per culture run.

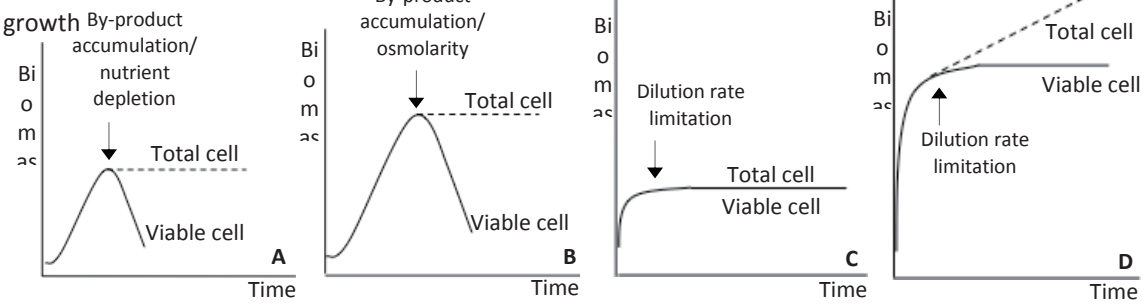


Figure 3.4. Schematic comparison of the typical cell growth profile depending on the implemented cell culture strategy.

As previously stated, the main objective of the work presented in this first chapter, was to increase the cell density in order to maximise the biocatalyst concentration. After the initial screening of media and supplements, a significant increment on Xv_{max} was accomplished. Nevertheless, we thought it was possible to explore the possibility of further improving the bioprocess. Hence, the study of two different cell culture strategies facing two different hypotheses was carried out. As mentioned before, a depletion of an essential nutrient might be the cause of the halt on cell culture propagation in batch cultures. To evaluate this hypothesis, punctual feeding of different nutritional supplements recommended for HEK293 cell line was tested. This strategy was tested as a preliminary approach of a controlled fed batch operation in bioreactor platform. It is also well known that some by-products (mainly, lactate and ammonia) are detrimental for cell growth at certain concentration. In this direction, complete media replacement was also studied as an approach for avoiding toxic metabolite accumulation. This strategy was considered as a manually perfusion strategy. The two cell culture strategies were evaluated in parallel at lab scale (125mL shake flask), and the obtained results are presented below.

3.3.1. PUNCTUAL FEEDING

3.3.1.1. SINGLE ADDITION: SCREENING OF NUTRITIONAL SUPPLEMENTS.

It has already been mentioned that the composition of the selected media is restricted, so the definition of which nutrient could be depleted and provide it at the required rate would have been unfeasible. Therefore, the punctual feeding strategy evaluation was done using commercial nutritional cocktails specifically designed for mammalian cell lines. Addition of nutritional supplements has already been explored in a prior section, but at this point it was decided to increase the number of supplements to evaluate, as each one has a different defined (but restricted) composition. The selected supplements were Cell Boost 1 (CB1), Cell Boost 5 (CB5) and Cell Boost 6 (CB6). These supplements have been specially conceived for punctual feeding or fed-batch strategies so they are designed as concentrated solutions of different nutrients. Specifically, CB5 and CB6 contain amino acids, vitamins, glucose, trace elements, growth factors, lipids and cholesterol, while CB1 contains only amino acids, vitamins and glucose.

In Figure 3.5 (left panel) cell growth and viability profiles of cell cultures in which punctual feeding was performed are depicted. The addition of the supplements was performed at the end of the exponential growth phase (established at around t=96h on basis to prior presented results) with the objective of increasing specific growth rate and/or extending the exponential growth phase resulting in an increment on the final viable cell density.

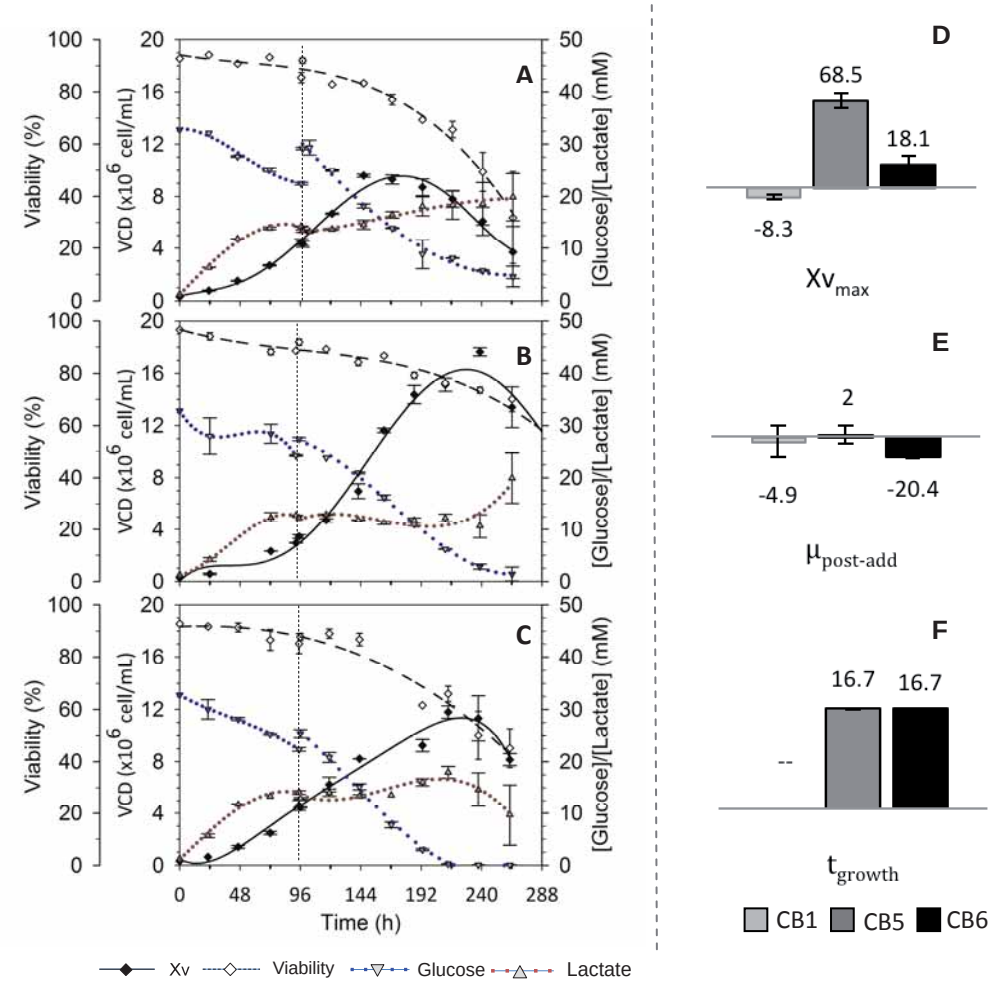


Figure 3.5. Effect of nutritional supplement addition on cell growth on HyQ SFMTransFx-293 + 5% (v/v) FBS. Left panel: Cell growth, viability, glucose and lactate concentration profiles of (A) CB1 (B) CB5 and (C) CB6. The average of two cell count of three biological replicates are shown. Error bars represent the standard error of the replicates. Right panel: Differences on cell growth parameters after normalization in respect control cell cultures (i.e. no Cell Boost addition). (D) Maximal viable cell density (E) growth rate estimated after 96h of cell culture (F) period of cell growth. Numbers on the top of the bar refer to the difference percentage in respect control cell

Firstly, it can be noticed that CB1 did not interfere significantly on cell growth whereas CB5 and CB6 addition resulted in an increment of Xv_{max} in respect no supplement addition (68% and 18%, respectively) (Figure 3.5d). Especially remarkable was the increasing on cell density with CB5 addition, reaching cell densities as high as $17.34\pm0.31x10^6$ cell/mL. In respect the effect of supplementation on μ_{max} and $t_{\mu max}$, none of the supplements could sustain the maximal growth rate beyond the first 96h of cell culture. The most noticeable drop on this parameter was detected on cultures in which CB6 was added, where growth rate after 96h was about the half in comparison to this parameter before the addition of the supplement. For the rest of cultures (no addition, CB1

addition and CB5 addition) growth rate dropped around 30% after 96h. Consequently, when comparing growth rate beyond 96h of supplemented cell cultures with no supplementation (Figure 3.5e), only cell cultures with CB1 and CB5 supplementation resulted therein the same range of no supplementation cell cultures, while growth rate after 96h was lower when CB6 were added in comparison to control cell cultures. However, when the latter supplement was boosted, a prolongation of cell growth period (24h longer in respect control cell cultures) was observed. This fact would explain the increment on Xv_{max} with CB6 supplementation, despite the higher drop on μ_{max}.

Taking all these data into account, we concluded that a depletion or limitation of some nutrient was occurring and the supplement addition that compensated better this limitation was CB5. Also, the drop on growth rate regardless the nutritional supplementation suggested that other factors, such as accumulation of toxic metabolites could be occurring. Finally, it can be noticed that when CB5 or CB6 was added, at the time of maximal cell density glucose was almost or completely depleted. This fact raised the hypothesis that a second addition of nutritional supplement could extend cell culture propagation. For the evaluation of this new hypothesis only CB5 was selected, as the best results in terms of cell growth were obtained with this supplement.

3.3.1.2. CELL BOOST 5 CONCENTRATION SCREENING.

Prior to the evaluation of additional nutritional supplementation, a toxicity study of CB5 was performed. This was made on basis that, for all the experiments presented so far ACDF-supplements were prepared at 40g/L, following manufacture's recommendations. Nevertheless, the limit of solution encountered for CB5 was the double (80g/L). As it was desired to use the supplement for fed-batch strategies, the next logical step was to test possible toxicity of the supplement if added at higher concentrations, in order to maximise as possible supplement concentration and consequently minimise volume increment. Main results obtained from cell cultures in which a punctual addition at t=96h of CB5 at 40g/L, 60g/L or 80g/L was done are resumed in Table 3.4. All cell cultures performed equally after CB5 addition, meaning that there are no toxic effects related to higher nutritional boost. Consequently, it was decided to use the highest concentration of CB5 for further experiments in order to minimise the volume increment in a punctual feeding or fed-batch strategy. It is worth to point out that the same viable cell density was achieved regardless cell boost concentration.

Table 3.4. Cell growth main important parameters of cell cultures supplemented at different CB5 concentrations

		Xv_{max} ($x10^6$ cell/mL)	t_{Xvmax} (h)	Viability (%)	μ_{max} ($x10^{-2}$ h^{-1})	$t_{\mu max}$ (h)	$\mu_{post\ ad}$ ($x10^{-2}$ h^{-1})
[CB5] (g/L)	**40**	16.64±1.02	240	86.24±3.66	2.62±0.04	95	1.77±0.19
	60	16.32±1.19	240	86.62±3.10	2.68±0.04	95	1.56±0.23
	80	17.01±1.61	240	86.61±2.68	2.49±0.22	95	1.73±0.16

3.3.1.3. CELL BOOST 5 DOUBLE ADDITION

Unique and double additions of CB5 at the new concentration encountered (i.e. 80g/L) were performed in parallel. The additional second feeding was of the same volume as the initial supplementation and it was performed 24h later. In Figure 3.6 the results of both cultures are presented. The additional feeding of the supplement did not contribute to increase the final cell density and in both cases (one addition and two additions) maximal viable cell density was around $18x10^6$ cell/mL. The second feeding neither had a significant effect on cell growth rate. After 96h, a similar drop of μ_{max} was detected in all cultures and cell expansion followed a linear pattern until 190h of cell culture (Table 3.5).

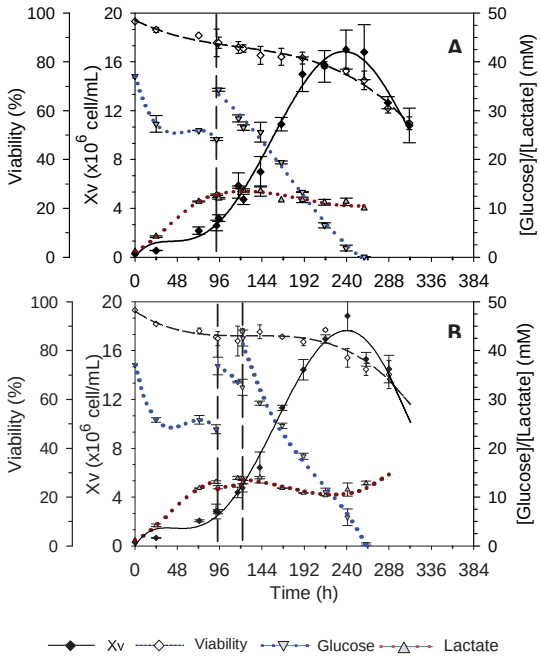


Figure 3.6. Evaluation of the effect of one or two feeding points on cell growth. A) Sole punctual feeding B) Two punctual feedings. Dashed lines indicate the moment of the addition.

Table 3.5. Cell growth parameters of cell cultures depicted in Figure 1.6.

	Xv_{max} ($x10^6$ cell/mL)	μ_{max} ($x10^{-2}h^{-1}$)	$\mu_{post\ ad}$ ($x10^{-2}h^{-1}$)	t_{growth} (h)
1 addition	17.01±1.61	2.29±0.22	1.67±0.23	190
2 addition	18.85±1.20	2.46±0.18	1.87±0.18	190

It is interesting to point out that regardless the number of feedings a significant drop on glucose consumption rate after the first feeding was noticed. The magnitude of this decrement was the same in all cell cultures and consequently, at the time of maximal cell density, the remaining glucose in cell media was higher on those cultures in which a second addition of CB5 have been performed. This fact indicates that the halt on cell growth was not related to a limitation of the main carbon source as it was hypothesized from the results of the unique addition. Conversely, the limitation encountered on Xv_{max} might be related to other factors such as the accumulation of toxic by-products, which will be addressed in the next section.

3.3.2. CELL MEDIA REPLACEMENT.

The second cell culture strategy evaluated was the complete medium replacement as an approach of a perfusion strategy. This approach allows removing any toxic metabolite accumulated in cell media that could be limiting cell growth. Moreover, the addition of fresh media would supply nutrients that could be depleted or in limiting concentration.

As it has been presented in the first section of this chapter, maximal growth rate of HEK293 cells growing in SFMTransFx+5%FBS (which was used for the study of media replacement) was maintained only until 96h of cell culture. With the aim of maintaining cells growing in exponential phase for longer time, the first media replacement was performed at that point of cell culture. As shown in Figure 3.7 this action enabled to extend μ_{max} phase for another 24 hours resulting in a 1.5-fold increment on final viable cell density (from 7.92±1.19x10^6 cell/mL to 12.19±1.07x10^6 cell/mL) (Table 3.6). 48 hours after this first media replacement, a slight decrement on cell growth rate was detected and a second media replacement was done. Comparing Figure 3.7e (no media replacement) with Figure 3.7g (two media replacements), it is noticed that the addition of fresh media succeeded on keeping cells growing exponentially for 48 additional hours (until 171 hours, which represents an overall elongation of exponential growth phase of 60% in respect of the initial scenario (i.e. no media replacement)). As a result, maximal cell density was also greater than cell cultures with one or none media replacement, reaching cell densities of 15.17±1.6x10^6 cell/mL.

Based on glucose and lactate profiles, it was decided to perform a third media replacement, but in this case μ_{max} was no longer maintained (Figure 3.7h) and Xv_{max} was not significantly incremented (Table 3.6).

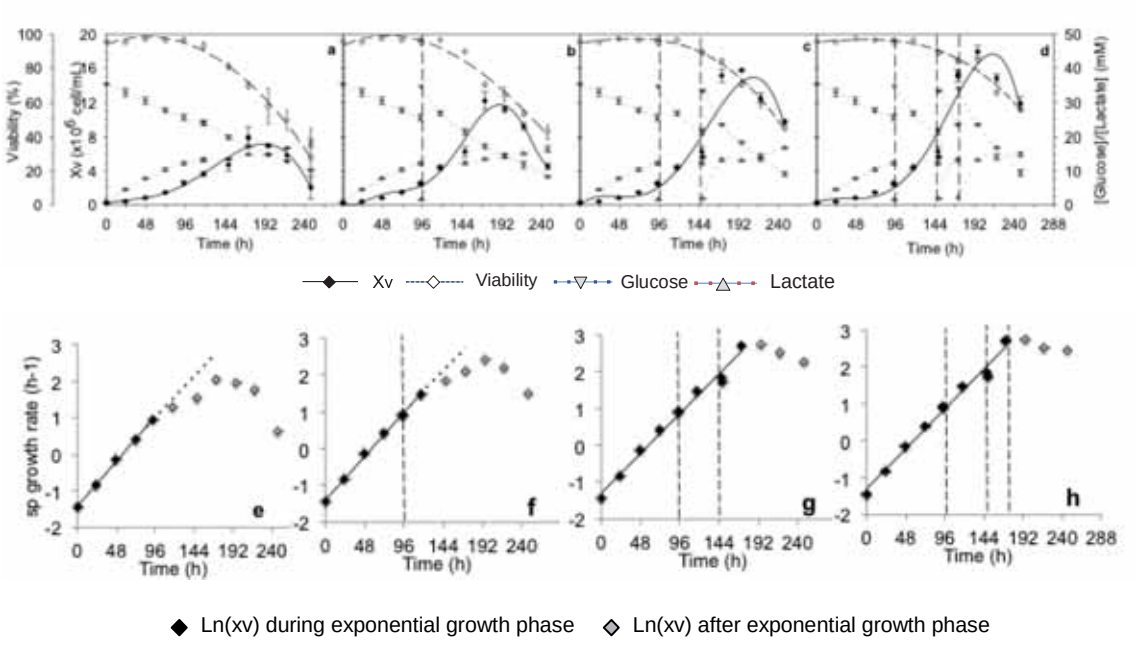


Figure 3.7. Profiles from cell cultures with complete media replacement (MR). Upper panel: Viable cell density, viability, glucose and lactate profiles of a) none MR (Ctrl); b) 1 MR; c) 2 MR and d) 3 MR. Lower panel: specific cell growth rate from e) none MR; e) 1 MR; f) 2 MR and g) 3 MR. Dashed lines indicate the time of media replacement.

Table 3.6. Maximal viable cell density, maximal specific cell growth rate and time of maximal growth rate of cell cultures presented on Figure 3.7.

	Xv_{max} ($x10^6$cell/mL)	μ_{max} ($x10^{-2}h^{-1}$)	$t\mu_{max}$ (h)
0MR	7.92±1.19	2.55±0.04	93
1MR	12.19±1.07	2.53±0.07	117
2MR	15.80±0.12	2.27±0.04	171
3MR	17.97±0.67	2.21±0.04	171

All this data allowed determining that although the accumulation of some metabolite was impairing cell growth, this was not the cause of the limitation on cell growth encountered on the experiments presented on section 3.3.1. By means of the media replacement strategy, the elongation of the exponential growth phase was achieved and this was not accomplished with the punctual feeding strategy. Nevertheless, the single addition of CB5 without the removal of possible toxic by-products, resulted in a Xv_{max} higher than the one obtained with one complete media replacement. Hence, the accumulated metabolites were not the main cause of cell growth halt and this phenomenon might be more related to the depletion of essential nutrients which are also supplied by the sequential media replacement.

Interestingly, maximal cell density achieved with media replacement strategy (17.97±1.5x10^6 cell/mL) was comparable to the one obtained with CB5 supplementation presented in section 3.3.1 (18.85±1.2x10^6 cell/mL), although the time needed to reach this cell density was reduced in 24h with the former strategy. This limit encountered with both strategies indicated that another factor different from cell media composition could be affecting cell growth and causing the stop on cell propagation. All the experiments presented so far were performed in shake flask, in which the oxygen is passively transferred to cell media. Therefore, the hypothesis of an oxygen limitation arose. In order to evaluate so, it was mandatory to change the cell culture platform from shake flask to bioreactor system. In our case, this change would also imply the scaling up of the process from lab scale (125mL shake flask) to bench scale (2L bioreactor).

3.3.3. BATCH REINFORCEMENT

The preliminary studies of media and supplementation screening (section 3.2) showed how the addition of CB5 and FBS at t=0h affected differently to cell growth. While FBS increased μ_{max} value, the supplementation with CB5 enlarged the exponential growth phase without affecting significatively to μ_{max} value in comparison to basal media. Therefore, it became interesting to study the "cross"-effect of supplementation with both components before moving on to bench scale bioreactors.

The selected concentration for FBS was 5% (v/v) in concordance with the results obtained in previous sections. Concerning CB5, those concentrations that at 40g/L without FBS (Figure 3.3) were no toxic and performed better (i.e. 5% and 10% v/v) were tested again but increasing the concentration to 80g/L. It was interesting to formulate a media as much nutritionally enriched as possible in order to fully exploit this nutritional content in bioreactor systems, where oxygen would be more effectively transferred to cell media.

In Figure 3.8 the results of the combination of both supplements are presented. Cultures with supplementation of CB5 at t=96h instead of t=0h were also included in the study as control of cell growth. These control cultures confirmed the previously presented results, as Xv_{max} of 17.96±0.68x10^6 cell/mL was achieved (Table 3.7). Besides, several relevant facts could be observed. Firstly, the supplementation of media with FBS and CB5 simultaneously did not have any toxic effect on cell growth. Specific growth rate was unaffected and maximal cell density was comparable to the sequential addition of the supplements. Also, cells could grow similarly regardless the concentration of CB5, so a possible impairment on cell growth due to osmotic pressure could be discarded. Interestingly, the addition of both supplements did not result in the prolongation of exponential

growth phase observed with the unique addition of CB5. These results are in good concordance with the ones obtained while exploring the punctual feeding strategy (section 3.3.1, FBS present in media) in which μ_{max} could not be maintained regardless the addition of nutritional supplement. This fact might indicate that the metabolism of HEK293 is different depending on the presence or absence of FBS in media resulting in this change on μ_{max} around 100 hours of cell culture. Finally, the concurrent addition of supplements enabled to reach cell densities as high as the ones achieved with punctual feeding and cell media replacement but with an easier cell culture strategy (i.e. reinforcement of cell culture media). This was remarkably important, as it was now possible to start the scaling up studies with a reformulated media that could push to the limit cell growth at bench scale.

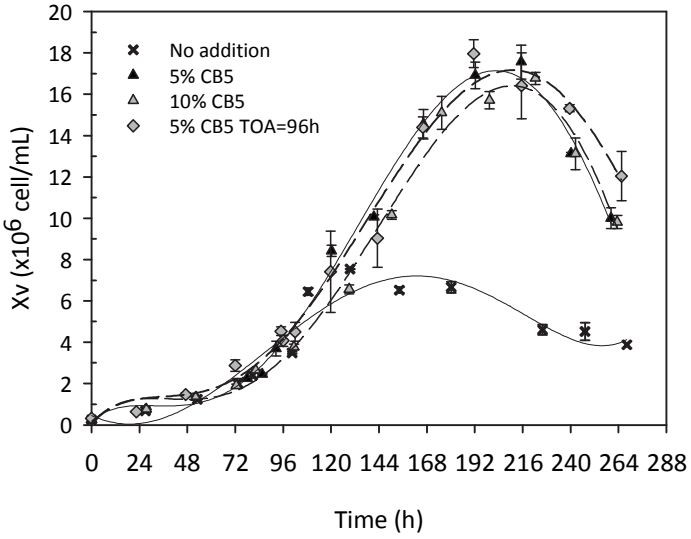


Figure 3.8. Cell growth profiles of cell cultures with simultaneous or sequential nutritional supplements addition (FBS and CB5). Reference cell culture without CB5 addition is also depicted. The average of two cell count of three biological replicates are shown. Error bars represent the standard error of the replicates.

Table 3.5. Main cell growth parameters corresponding to cell cultures presented in Figure 3.8.

%CB5 (v/v)	TOA (h)	Xv_{max} ($x10^6$cell·mL^{-1})	μ_{max} ($x10^{-2}$ h^{-1})	Viability to Xv_{max} (%)	T μ_{max} (h)
0	--	7.54±0.03	2.69±0.30	87.95±4.74	100
5	0	17.56±0.82	2.66±0.18	93.07±1.57	100
5	96	17.96±0.68	2.84±0.61	90.72±2.68	100
10	0	15.77±0.29	2.72±0.07	91.58±0.57	100

3.4. STIRRED TANK BIOREACTOR STUDIES

3.4.1. BATCH OPERATION

The prior study of cell culture operations approach at lab scale allowed us to define the initial point for the study of cell growth in bioreactor. Initially, we evaluated cell growth in batch operation using HyQSFMTransFx-293 4mM glutaMAX with 5% (v/v) FBS and 10% (v/v) CB5 (80g/L) supplementation from t=0h. The main objective was to asses if oxygen limitation was the cause of the limit encountered on Xv_{max} when working with shake flask. Besides, it is widely accepted that cells behave differently depending on cell culture platform in which they are grown. Several parameters such as mass transfer rate, homogeneity of mixing, mixing time and shear stress differ from shaking platforms to stirred platforms [15,16] and this might influence growth and metabolism of cultured cells. Each microorganism is differently affected by these parameters and the possible differences on cell growth depending on cell culture platform must be evaluated for each case. Consequently, the characterisation of HEK293 cell growth in this platform and its comparison to shake flask cell culture should be addressed.

The results of a batch cell culture in 2L STR-bioreactor (working volume 1.5L) and a batch cell culture performed in parallel in a 1L shake flask (working volume 250mL) are presented in Figure 3.9 and Figure 3.10. The set point values for the culture carried out on STR bioreactor are resumed in Table 3.8. The outcome of both cell cultures was compared in terms of cell growth and metabolic behaviour.

Table 3.6. Set points for controlled variables of batch STR cell culture presented in Figure 3.9.

	pH	T (ºC)	pO2 (%)	Agitation (rpm)
Set point	7.1	37	60	80

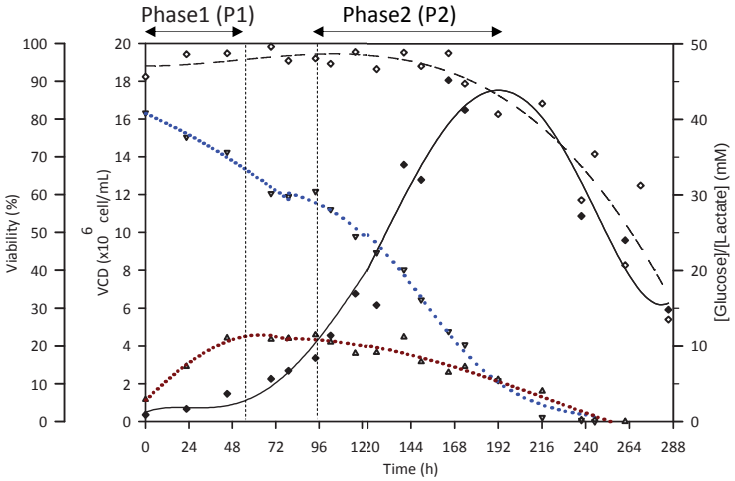


Figure 3.9. HEK293 batch cell culture in 1L-shake flask (cell culture control)

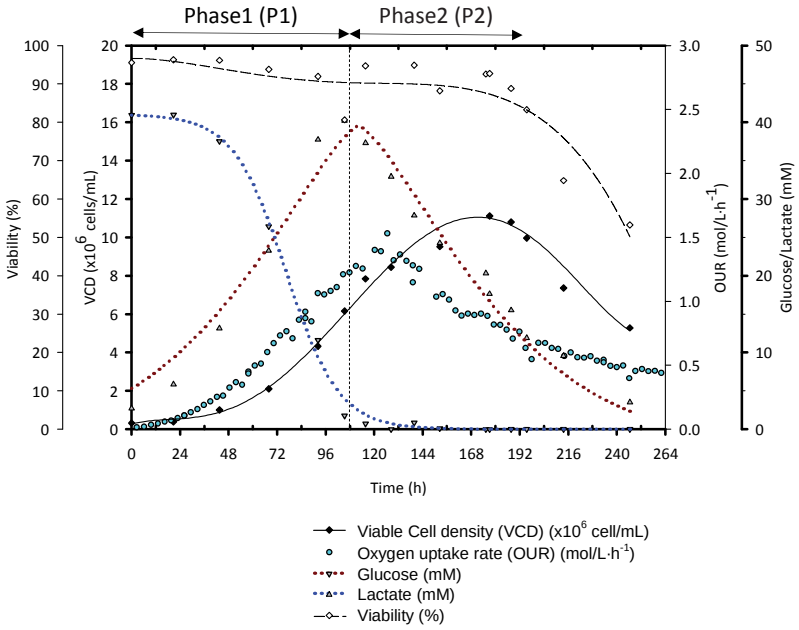


Figure 3.10. HEK293 cell culture in STR-bioreactor in batch operation. Cell growth, viability, glucose, lactate and Oxigen Uptake rate (OUR) profiles are indicated. Cell culture media used was HyQSFMTransFx-293 supplemented with 4mM glutaMAX, 5%FBS and 10%CB5 (80g/L)

First of all, a significant drop on Xv_{max} was detected in batch cultures when changing from shake flask to STR platform. In shake flask, HEK293 cells grew until reaching a maximal viable cell density of $18.1x10^6$ cell/mL (Figure 3.9 and Table 3.9), but in stirred tank bioreactor the highest value for viable cell density was $11.4x10^6$ cell/mL (Figure 3.10 and Table 3.9). Moreover, exponential growth was observed for cells from t=0h in shake flask while a lag-phase of 24h was noticed in the culture performed in bioreactor. Additionally, cell aggregation was observed onwards 157h of cell culture in bioreactor platform but not in shake flask. All these facts indicate that shear stress was probably

affecting cell growth: cells needed an adaptation to the hydrodynamic forces inside the bioreactor resulting in the lag phase and in order to protect themselves to stirring and bubbling damage, cells tend to aggregate as cell culture progressed.

Nevertheless, the impairment on cell growth in comparison to shake flask cultures could not be only related to shear stress as deducted from the analysis of viability and OUR profiles (Figure 3.10). Cell viability was sustained over 85% until maximal cell density was reached and OUR value continuously incremented until t=120h (i.e. approximately 36h before cell aggregation was noticed). It is worth to mention that OUR exponential profile was only noticed from 20 to 77hours of cell culture, which is approximately 30 hours less than the estimated exponential growth phase (Table 3.9). Thereafter, the increment of OUR value was linear instead of exponential during additional 48 hours and it finally change to a constant drop until the end of cell culture. This is in good concordance with studies previously published[17,18] and confirms that OUR value can be successfully used as an on-line measurement for cell culture monitoring. Interestingly, the end of exponential growth phase occurred concomitantly with glucose depletion in media, the triggering of lactate switch from production to consumption and a decreasing OUR profile. This fact might indicate that the halt on cell growth in bioreactor platform was related to the depletion of the main carbon source from media.

Even more interesting was the complete different metabolic behaviour encountered in shake flask in comparison to the metabolism observed in bioreactor cultures. In the latter platform, the lactate shift from production phase (named phase 1 or P1) to net consumption phase (referred to as phase 2 or P2) and its use as carbon source was clearly linked to the depletion of glucose from media as it was expected[19–21] In the case of shake flask cultures, a net lactate consumption was observed in presence of glucose, being glucose concentration far from limiting values ($[gluc]_{t=102h}$=28.04mM)[22]. In other words, a concomitant consumption of glucose and lactate was detected in shake flask while a sequential consumption of glucose and lactate was observed in bioreactor. Besides, the dynamics of the switch from lactate production to consumption were different between the two cell platforms: the metabolic change was quite sudden in STR-bioreactor while a “transition-phase” (i.e. neither lactate secretion nor lactate uptake from media) of about 30h was detected in shake flask cultures. Remarkably, in shake flask cultures the shift on lactate metabolism from P1 to P2 was observed when lactate concentration was around 12mM. This maximal lactate accumulation was also observed in prior experiments in shake flask, such as the studies of cell culture strategies, both in punctual addition and in cell media replacement (Figure 3.6 and Figure 3.7, respectively). In regard to the specific consumption and production rates, some differences were also encountered when comparing cultures performed in different platforms (Table 3.9). Specific consumption rate (q_{gluc})

during P1 in bioreactor was 1.6-fold higher than in shake flask, whereas specific lactate production rate (q_{lac}) during this phase were comparable for both platforms. This would indicate that cells present a more efficient glucose metabolism when cultured in bioreactor as more glucose molecules are completely oxidized instead of undergoing anaerobic glucose respiration resulting into lactate formation and secretion. Nevertheless, it must be borne in mind that P1 was longer in bioreactor than in shake flask resulting into a higher final concentration of lactate in cell media. Concerning P2, a significant drop on q_{gluc} was detected regardless the cell platform, but whereas this drop is related to glucose depletion in STR-bioreactor, in shake flask must be correlated to a parameter different from glucose limitation. Lactate uptake rate was greater in bioreactor during P2 in comparison to shake flask, which is in good correlation with the fact that in the latter platform two metabolites (glucose and lactate) are consumed whereas in bioreactor glucose was already depleted. The phenomena of glucose and lactate concomitant consumption and the differences on metabolic rates encountered depending on cell culture platform will be addressed in detail in Chapter 4 of the present doctoral thesis.

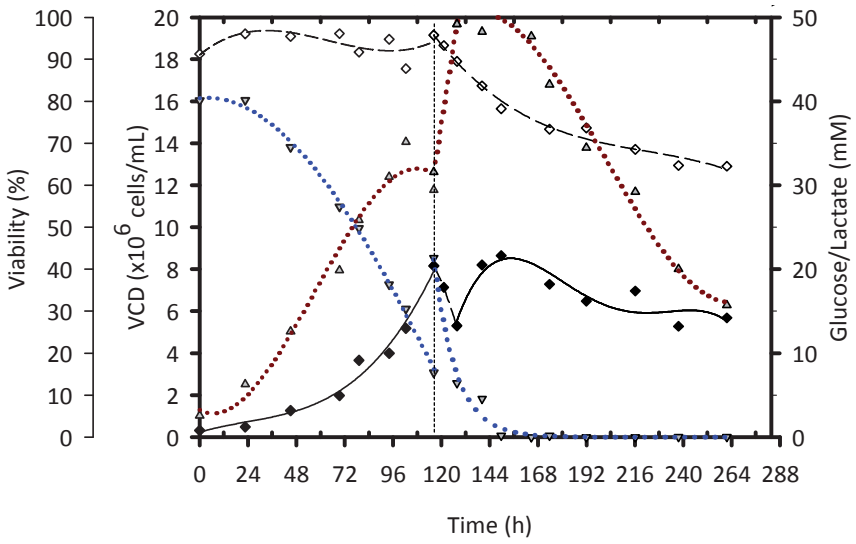
Table 3.7. Growth and metabolic kinetic parameters of cell cultures depicted in Figure 3.9. and in Figure 3.10.

	STR-bioreactor	Shake flask
Xv_{max} ($x10^6$ cell/mL)	11.44	18.08
μ_{max} ($x10^{-2}h^{-1}$)	3.12	2.39
$t_{\mu max}$ (h)	116	102
qO_2 ($nmol/10^6 cell \cdot h^{-1}$)*	321.6	ND
t_{OUR_exp} (h)	77	ND
$q_{gluc\ Ph1}$ ($nmol/10^6 cell \cdot h^{-1}$)	220.01	136.58
$q_{lac\ Ph1}$ ($nmol/10^6 cell \cdot h^{-1}$)	245.64	217.67
$q_{gluc\ Ph2}$($nmol/10^6 cell \cdot h^{-1}$)	2.136	26.20
$q_{lac\ FPh2}$($nmol/10^6 cell \cdot h^{-1}$)	-36.45	-5.82

3.4.2. FED-BATCH OPERATION

In order to assess if the limitation on cell growth observed in batch operation in STR was due to the depletion of glucose, punctual nutrient supplementation strategy was evaluated. The basal media used was the same than in batch-mode operation (i.e. SFMTransFx-293 4mM glutaMAX 5%FBS 10%CB5 (80g/L)) and the addition performed was 10%v/v of CB5 (80g/L) Based on previous batch operation the feeding was when μ decreased, which was at a glucose concentration of about 7mM. Shortly after the addition, severe cell lysis was observed and consequently cell culture viability sharply dropped (Figure 3.11). Cells that outlived the supplement addition grew linearly for additional 24h, but thereafter an irreversible death phase of the cell culture occurred. These results led us to consider that the addition of CB5 to a media that contains a relatively high concentration of nutrients and by-products might cause a significant increment on osmolarity resulting in an osmotic shock and cell lysis.

Figure 3.11. Punctual addition of CB5 on HEK293 cell culture in SFMTransFx-293 supplemented with 4mM glutaMAX, 5% FBS and 10% Cb5 (80g/L).



After these results, it was considered to further evaluate fed-batch strategy but the removal of CB5 from the basal media was performed in order to reduce the osmolarity. Also, the feeding media was reformulated and the strategy of punctual feeding was changed to a step-wise automatic feeding strategy. Thus, in the second fed-batch approach the initial media was SFMTransFx 4mM glutaMAX 5%FBS, the feeding media was as detailed in Materials and Methods chapter (section 8.2.1.3) and the predefined feeding profile was automatic and controlled by the DCU. The feeding control loop

was programmed using MFCS/Win 3.0 software and was based on the formula deduced from the mass balance and described in Eq.3.2. Specific growth rate was defined at 80% of μ_{max} in order to avoid accumulation of glucose and lactate in media. μ_{max} was calculated during the batch phase of cell culture. The onset of feeding was manually performed when a change on OUR tendency was detected. The set points for the controlled parameters (i.e. Tº, pH, stirring) were the same as in batch mode operation (Table 3.8). In order to correctly evaluate the effect of the fed-batch strategy selected, a batch culture was carried out in parallel using the same basal media as in the fed-batch culture (i.e. HyQ SFMTransFx 4mM glutaMAX 5%FBS). The results are depicted in Figure 3.11 and Figure 3.12 (batch and fed-batch, respectively).

$$V_{ad} = \frac{1}{S_0} \cdot \left(\frac{m_{sx}}{\mu_{sp}} + \frac{1}{Y_{XS}} \right) \cdot X(t) \cdot V(t) \cdot \left(e^{(\mu_{sp} \cdot \Delta t)} - 1 \right) \qquad \text{Eq. 3.2.}$$

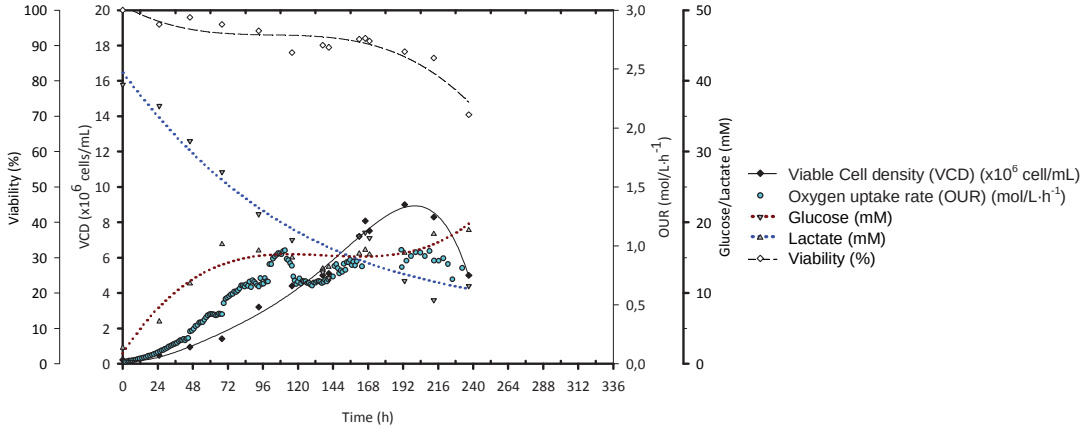


Figure 3.11.HEK293 cell culture in STR-bioreactor in batch operation. Cell culture media used: HyQSFMTransFx-293 4mM glutaMAX+5%FBS.

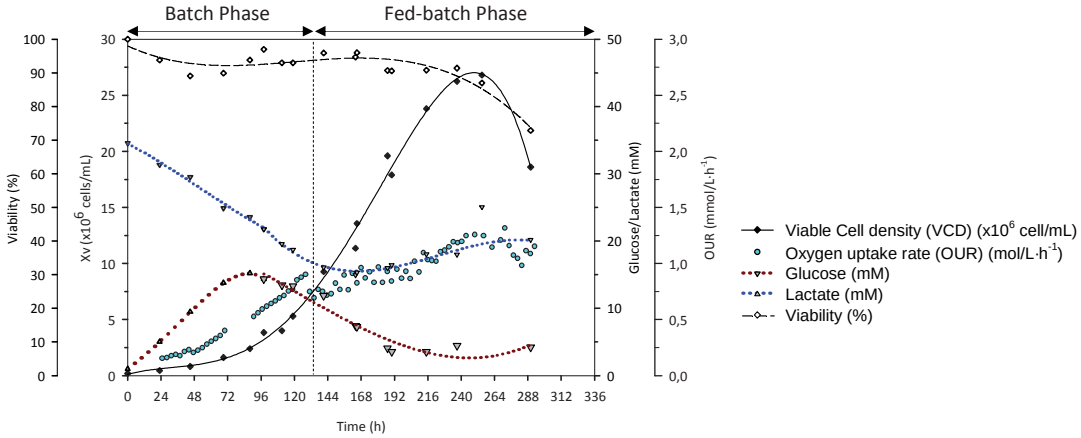


Figure 3.12. HEK293 cell culture in STR-bioreactor in fed-batch operation. The dashed line indicates the onset of fed-batch.

From the comparison of cell growth between the studied strategies, it can be noticed that the implementation of fed-batch strategy succeeded on the objective of increasing Xv_{max}, enabling to overcome the limit encountered at $18x10^6$ cell/mL. Specifically, Xv_{max} was $26.8x10^6$ cell/mL, which represents more than 3-fold increment on this parameter in comparison to batch mode operation performed in the same media (Table 3.10). During the batch phase, μ_{max} of both cultures were comparable ($2.62x10^{-2}h^{-1}$ and $2.75x10^{-2}h^{-1}$). With the onset of the feeding, specific growth rate slightly decreased but it could be maintained at $2.03\ x10^{-2}h^{-1}$ for additional 70h. Hence, it can be said that cells grew almost exponentially for 165 hours of cell culture and thereafter, cells continued growing linearly until t=240h when the start of cell death was observed. On the contrary, specific growth rate of cells in batch culture sharply slowed down beyond 96h. These differences on cell growth show that beyond 96h some nutritional limitation is occurring in batch operation with the basal media used and a correct feeding of nutritional supplementation can compensate it. Also, the hypothesis of oxygen limitation in shake flask platform has been validated as cells can achieve higher cell densities when cultured in a platform that permits better mass transfer from the gas to liquid phase. It should be highlighted that to our knowledge, the cell density here presented has never been reported before in HEK293 cell line even with more intense cell culture strategies (Appendix 10.1).

Table 3.8. Growth and metabolic kinetic parameters of cell cultures depicted in Figure 3.12 and Figure 3.13

	Batch-operation	Fed-Batch operation (Batch phase)	Fed-Batch operation (Fed-batch phase)
Xv_{max} ($x10^6$ cell/mL)	8.3	4	26.8
μ ($x10^{-2}h^{-1}$)	2.62	2.75	2.03
t_μ (h)	116	111	165
qO_2 ($nmol/10^6cell{\cdot}h^{-1}$)	200.3	201.3	ND
$q_{glucPh1}$ ($nmol/10^6cell{\cdot}h^{-1}$)	246.07	117.96	-4.29
$q_{lac\ Ph1}$ ($nmol/10^6cell{\cdot}h^{-1}$)	290.09	149.69	-1.29
$q_{gluc\ Ph2}$ ($nmol/10^6cell{\cdot}h^{-1}$)	44.23	48.19	--
$q_{lac\ Ph2}$ ($nmol/10^6cell{\cdot}h^{-1}$)	-12.39	-21.26	--

3.5. CONCLUSIONS

After an intense study of HEK293 cell growth under different cell media and implementing different cell culture strategies, such as batch, media replacement and fed-batch, more than 10-fold increment on Xv_{max} was successfully achieved. Cells cultured in media recommended by the cell line providers grew only until $2.1x10^6$ cell/mL. The implemented fed-batch strategy allowed to largely overcoming this value and reaching a viable cell density of $26.8x10^6$ cell/mL. Hence, one of the two parameters affecting volumetric productivity (Eq.3.1) has been highly maximised. With the promising results obtained so far, perfusion cell culture operation could have been addressed as it is already stated that this strategy results in higher cell densities than fed-batch operation (Figure 3.4). Nevertheless, at this point of the study, it was decided to move towards addressing the increment of the second parameter affecting volumetric productivity (i.e. q_p), by the study of different bioprocesses, which are presented in following chapters.

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