Characterization of Insect Cell Lines Is Required for Appropriate Industrial Processes

Case Study of High-Five Cells for Recombinant Protein Production

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The Insect Cell - Baculovirus Expression Vector System (IC-BEVS) is widely used for the production of complex recombinant (glyco)proteins. The simplicity of insect cell cultivation in suspension serum-free media and the easy construction of recombinant baculovirus vectors have made the BEVS quite an effective expression system. On the other hand, the BEVS is a transient lytic system that may present some drawbacks in purification and potential degradation of the products. Among the various insect cell lines, the High-Five cell line has a great potential for the production of recombinant proteins using the BEVS in stirred bioreactors, reaching high cell densities and high protein production levels. Moreover, these cells can tolerate environmental stresses and can be cultivated on a large scale (Chapter 1). Unfortunately, up to now, there have been limited data available regarding suitable culture conditions and the metabolism of High-Five cells, a key requirement for the rational development of new processes.

The overall goal of the present work was the study of these High-Five cells, in order to develop sophisticated new processes as alternatives to batch cultivation. The original contributions have been developed along two axes. The first axis concerns the study of the physiology and metabolism of High-Five cells. At first, we undertook a study aiming to prevent cell ring formation on suspension culture recipient walls (Chapter 2). Next, we analyzed environmental factors affecting insect cell growth and death, by comparing and developing methods able to distinguish between apoptosis and necrosis of cells (Chapter 3). The comprehensive study of the extended metabolism of High-Five cells was done using a metabolic flux network that takes account of the catabolism but also the anabolism of uninfected and baculovirus-infected cells (Chapter 4).

The second axis was the application of the previously gained knowledge on High-Five cells to develop high-density systems specifically adapted to them: a fed-batch feeding strategy consisting of different pulses developed to increase the productivity of cells during infection (Chapter 5) and a fixed-bed reactor system (Chapter 6), as an alternative to classic perfusion, adapted to High-Five cells for recombinant protein production.

In sum, new physiological and metabolic knowledge has been translated into new process options for High-Five cells.

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CHARACTERIZATION OF INSECT CELL LINES IS REQUIRED FOR APPROPRIATE INDUSTRIAL PROCESSES

CASE STUDY OF HIGH-FIVE CELLS FOR RECOMBINANT PROTEIN PRODUCTION

Dissertation présentée en vue de l'obtention du titre de
Docteur en Sciences Agronomiques et Ingénierie Biologique
Par Drugmand Jean-Christophe

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À Marie-Hélène,

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Abstract

The Insect Cell - Baculovirus Expression Vector System (BEVS) is widely used for the production of complex recombinant (glyco)proteins. The simplicity of insect cell cultivation in suspension serum-free media and the easy construction of recombinant baculovirus vectors have made the BEVS quite an effective expression system. The baculovirus has a small genome, is quite efficient to manipulate and is non-pathogenic. On the other hand, the BEVS is a transient lytic expression system that may present some drawbacks in purification of the recombinant protein and potential degradation of the products. Among the various insect cell lines, the High-Five cell line has a great potential for the production of recombinant proteins using the BEVS in stirred bioreactors, reaching high cell densities and high protein production levels. Moreover, these cells can tolerate environmental stresses and can be cultivated on a large scale. Unfortunately, up to now, there have been limited data available regarding suitable culture conditions and the metabolism of High-Five cells, a key requirement for the rational development of new processes.

The overall goal of this work was the study of the physiology and the metabolism of High-Five cells for development of processes. We worked in parallel with the Sf-9 and High-Five insect cell lines to compare our new results on High-Five cells to the available literature on the well-known Sf-9 cells. Cells were adapted and cultivated in our own serum- and protein-free YPR medium to which we added dextran. Two recombinant *Autographa californica* baculoviruses encoding the model proteins, glycosylated secreted alkaline phosphatase (seAP) and β-galactosidase (β-gal) (a cytosolic protein), were employed in infection studies.

When cultivated in suspension cultures, High-Five cells tend to produce cell ring on shake-flask and bioreactor vessel walls. This cell ring, mainly composed of dead cells, is deleterious for the cultivation and contributes to a decrease in the freely suspended cell density and in the overall viability. We were able to prevent the formation of a cell ring by addition of an optimal type (67 kDa) and concentration (1 g/l) of dextran to the medium. Hence, we recommend using this supplemented medium for High-Five cells in suspension culture which allows to reach ca. 9-10x10^6 cells/ml in batch with a high growth rate (μ = 0.025 h^{-1}). The adsorption of dextran simultaneously on the recipient walls and on cell membranes appears to inhibit the complex cumulative and indirect effect of cell ring formation.

Next, we analyzed environmental factors affecting insect cell death. First, we developed and compared methods able to distinguish between apoptosis and necrosis of insect cells. Then, we used them, to understand what conditions cause cell death in High-Five cultures. Among these, moderate physical stresses (increase of shear stress, absence of

Pluronic F-68) triggered a shortening of the growth phase and caused High-Five cell necrosis, but did not affect apoptosis. On the contrary, moderate metabolic stresses such as the removal of hydrolysates (Yeastolate and Primatone), the deprivation of glucose or the presence of ammonia, seemed to induce apoptosis. The presence of lactate did not seem to cause apoptosis of High-Five cells.

A comprehensive study of High-Five cell metabolism was made using a metabolic flux network. Prior published information on insect cell metabolism was used to build the metabolic pathway network operating in these cells. Extracellular consumption and production of metabolites were directly used in the flux network as catabolic pathways, while the biomass composition of the cells was used jointly with literature data to determine the anabolic intracellular fluxes. Such a methodology made possible the construction of global pathway maps of High-Five and Sf-9 cells under various environmental conditions. Differences were thus, revealed in the central metabolism between the two cell lines: High-Five cells have higher metabolic activities than Sf-9 cells; High-Five cells produce more lactate and ammonia and less alanine than Sf-9 cells; High-Five cells seem to consume preferentially asparagine whereas Sf-9 prefer glutamine; High-Five cells are able to consume lactic acid and alanine when glucose becomes deficient; High-Five cells do not consume the same amino acids as energy source as Sf-9 cells.

The final step was the application of the knowledge gained on physiology and metabolism of High-Five cells to develop high-density systems specifically adapted to them. A fed-batch feeding strategy consisting of different pulses was developed to increase the productivity of High-Five cells during infection. Our approach was based on an understanding of the cells' needs during infection and could be summarized in the following questions: what nutrient(s) is(are) needed to be brought in as additive(s) to the cells ? when do the cells need it(them) ? at what overall level (how much) ? how often ? and at what individual concentration ? We developed a High-Five feeding strategy consisting in pulses of media supplemented with glucose, glutamine, Primatone and Yeastolate whose formulations evolve as a function of cellular requirements. This sustains a recombinant protein production titer of β-gal and seAP 5.5 fold higher than the one obtained in batch infections without medium changeover.

The elaboration of a fixed-bed reactor system, as an alternative to the classical perfusion with cell retention system, adapted to High-Five cells for recombinant protein production was based on the research of the best environmental conditions: what is the best support? what is the best level of compactness ? what media ? what DO ? etc. We developed a High-Five packed bed bioreactor with BioNOC II (Cesco Bioengineering, Hsin-Chu, Taiwan) microcarriers that allows to sustain a density of 400.10^6 cells/g of support and a volumetric production titer of β-gal 2.5 fold higher that in batch. Moreover, we investigated the key controlling parameters for such cultivation (amino acid consumption rate, DO, pCO_2, glucose consumption rate and glucose concentration in the fresh medium, gradient of oxygen, ammonia and lactate accumulation and, most crucially, the pH).

In sum, new physiological and metabolic knowledge has been translated to new process options for High-Five cells.

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List of abbreviations

β-gal	β-galactosidase
AcMNPV	*Autographa californica* multiple nuclear polyhedrosis virus
AO	Acridine orange
B. mori	*Bombyx mori*
BEVS	Baculovirus Expression Vector System
Bm	*Bombyx mori* cell line
BmNPV	*Bombyx mori* nuclear polyhedrosis virus
BSA	Bovine serum albumin
BV	Budded virus
CDI	Cellular density at infection
CSTR	Continuous stirred tank reactor
D.Mel	*Drosophila melanogaster* cell line
DO	Dissolved oxygen
DIP	Defective infecting particles
Eth	Ethidium homodimer
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GV	Granulosis virus
h.p.i.	Hour post infection
HPLC	High-performance liquid chromatography
IC-BEVS	Insect Cell Baculovirus Expression Vector System
kd	Specific death rate
LDH	Lactate dehydrogenase
MNPV	Muliply embedded nuclear polyhedrosis virus
MOI	Multiplicity of infection
MW	Molecular weight
NPV	Nuclear polyhedrosis virus
ONPG	o-nitrophenyl-β-D-galactoside
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
OV	Occluded virus
PBS	Phosphate buffer saline
pCO_2	Partial pressure of dissolved CO_2
pfu	Plaque forming units
PI	Propidium iodide
q	Specific rate of consumption/production
Q	Volumetric rate of consumption/production
Rh123	Rhodamine 123
r-protein(s)	recombinant protein(s)
S.frugiperda	*Spodoptera frugiperda*
seAP	Secreted alkaline phosphatase
Sf	*Spodoptera frugiperda* cell line
STR	Stirred tank reactor
T.ni	*Trichoplusia ni*
Tni	*Trichoplusia ni* cell line
TOI	Time of infection
μ	Specific growth rate
YPR medium	Yeastolate-Primatone medium

Foreword

BIOTECHNOLOGY

For thousands of years, microorganisms have been used to aid us in the making and preservation of foodstuffs such as bread, beer, wine, vinegar, cheese and other substances resulting from fermentation. These sophisticated products were developed over many centuries to satisfy the palate and psyche of humans. Processes using microorganisms to manufacture non-food products were developed during World War I leading to acetone, butanol and glycerol needed for the production of munitions. After the war, bioconversion and enzymatic processes were developed, yielding many useful products such as amino acids, nucleotides, vitamins, organic acids, solvents and polysaccharides. The term "biotechnology" was defined to designate biology-based technology whose products are made with the aid of living organisms. The second phase in the evolution of biotechnology was the development and commercialization of pharmaceutical products such as antibiotics, vaccines, etc. The first one was penicillin, which was discovered in 1929 and was developed after World War II. The development of the biotechnology field expanded into agricultural (biopesticides, bioherbicides, etc.) and chemical applications (production of single isomers such as L-amino acids, etc.)

In the early of 1970s, the third phase of biotechnology development began with the birth of recombinant DNA technology. It has allowed primarily to produce biologically active and therapeutically useful proteins. The first monoclonal antibody was commercialized in 1982 as a diagnostic product. In 1983, human insulin ("humulin") became the first recombinant protein to be used as a human therapeutic (Demain, 2000). Today, the modern biotechnology industry is worth over €15 billion and has made a major impact in the biopharmaceutical and agricultural fields (Demain, 2000).

INSECT CELLS

Today, the areas of medicine and of biomedical science exhibit a demand for high amount of inexpensive and safe recombinant proteins. These can be produced in prokaryotic cells (bacteria), in eukaryotic cells (yeast, fungi, plant cells, mammalian cells, insect cells), in plant and animal (milk, eggs, insect larvae) multicellular organisms or in vitro in cell-free expression systems using biomolecular tools. Amongst these, animal cell culture is emerging as the most important technology for the expression of therapeutic or diagnostic glycoproteins and vaccines. Insect cells, and particularly lepidopteran cells, appear as an attractive alternative to mammalian cell culture for recombinant protein expression. Insect cells have been traditionally used for the production of biopesticides. However, since the early 1980's, the introduction of the **B**aculovirus **E**xpression **V**ector **S**ystem (BEVS) has rendered insect cells an important part of the expression systems for recombinant protein production.

This BEVS is a transient lytic expression system that consists in a co-culture of a recombinant baculovirus with in vitro propagated **I**nsect **C**ells (IC-BEVS) or in vivo infected insect larvae. Recombinant baculoviruses are constructed by replacing a non lethal viral gene by a foreign gene. These genes are generally expressed at the end of the infection cycle. So, the recombinant proteins are expressed at the end of the virus cycle and harvested after or shortly before the lysis of the cells.

This more and more widely used expression system has the advantage to produce high amount of recombinant proteins with complex post-translational modifications in a short time. Moreover, the recombinant baculoviruses that consist of double-stranded circular DNA are genetically very stable. Insect cells and baculoviruses are non pathogenic for humans (O'Reilly et al., 1992; Rhodes, 1996). Furthermore, insect cells can be cultivated at high scale in serum-free medium, which represents an important biosafety advantage. Today, more than 500 genes have been expressed in IC-BEVS and it is used by almost 50 % of the scientific papers that employ a eukaryotic expression system (Palomares et al., 2006). Its main field of application is the production of recombinant proteins for biomedical research. In industry, insect cells are used essentially to produce proteins needed for diagnostics. Therapeutic utilization imposes some requirements in addition to specific post-translational modifications: high levels of biosafety, high productivities as well as high quality standards that have to be maintained all along the production process and from

batch to batch. Nowadays, four commercial therapeutic veterinary products are expressed with this system (Schmidt, 2004; Walsh, 2005). Additionally, more and more corporate pharmaceutical groups are currently developing an interest to produce vaccines or therapeutics using IC-BEVS, and many of them are today in clinical phase II and III trials (American Food and Drug Administration and European Medicines Agency).

The ever-increasing scientific and commercial interest in the BEVS can be illustrated by the opportunity offered by the BEVS to solve the bird flu crisis. Bird flu is a disease caused by an influenza type A, subtype H virus. Studies have suggested that the BEVS-expressed H5 hemagglutinin (HA) from the avian H5N1 influenza virus strain induced functional antibodies in individuals who had no prior exposure to the H5 virus (Cox, 2004). Nowadays, Protein Sciences (Meriden, CT) is planning to use in vivo caterpillar larvae infected with a recombinant baculovirus producing H5 hemagglutinin as veterinary vaccine against the bird flu at a price of € 3.5 cents per bird, available for 30 billion chickens in a few months (Protein Sciences, 2006). Besides, according to the World Health Organization (WHO), in case of mutation of the avian H5 virus and transmission to humans, insect cell cultures infected with BEVS could be one of the most efficient and powerful manufacturing technologies able to provide an appropriate solution to this adverse health care scenario. According to an internal report, Protein Sciences is in possession of several BEVS expression clones of influenza A subtype H. They are exploring the potential of the production of a human epidemiologic vaccine in BEVS currently in a clinical phase II trial.

HIGH-FIVE CELLS

Since the establishment of the first insect cell line in the 60's by Grace, various insect cell lines have been set up and different viruses have been used. However, it has only been a few years since insect cell lines able to satisfy industrial culture conditions have been established. They are able to grow in serum-free media with a good resistance to shear stress, osmotic shock, by-products accumulation and variations of pH, oxygen and CO_2. They can reach high cell density with a high growth rate and are capable of high production rates.

Among them, the Sf-9 and High-Five™ cell lines are the most commonly used. The Sf-9 cell line was established in 1983 by Smith et al. and was derived from a clone

of the ovarian Sf-21 line previously isolated from the butterfly *Spodoptera frugiperda*. The High-Five™ cells came from embryonic cells of the butterfly *Trichoplusia ni*. This cell line was patented in 1994 by Granados.

In the 80's and 90's, Sf-9 cells were the most productive and the most widely used insect cells. Their metabolism was widely studied during the 90's and it is well known today. Many media have been specifically developed for this cell line. Moreover, various fed-batch, fixed bed and perfusion processes involving Sf-9 cells have been developed. These cells are able to produce high amounts of viral particles, and allow to amplify baculoviruses in a straightforward manner. In the 90's, the majority of the industrial processes developed using IC-BEVS technology employed Sf-9 cells. Today, High-Five cells is considered to be the most productive insect cell line, up to ten times more productive than the Sf-9 cell line. Moreover, they have a superior capacity of secretion of glycoproteins. They are more robust than Sf-9 cells, less affected by by-product accumulation and by DO or pH shocks. They are quite able to be cultivated on a high scale. Unfortunately, fewer available media are specific to High-Five cells, their metabolism is less well known and fewer processes are developed for this cell line.

CONTRIBUTION OF THE PRESENT WORK

The objective of the present work is the study of the physiology and the metabolism of these High-Five cells for the development of processes for the production of recombinant proteins. The global goal is to better understand their metabolism and the environmental conditions affecting the cells, in order to apply these observations to advanced processes such as those involving fed-batch and fixed bed cultivations.

The main reasons for utilizing High-Five cells instead of other insect cell lines in an IC-BEVS process are discussed in detail in the literature review (Chapter 1). In this chapter, we have surveyed the existing knowledge on insect cell metabolism and the cultivation conditions of these cells. We have specifically addressed the metabolic knowledge as well as critically examined the process aspects of insect cell exploitation, including advantages and disadvantages of their cultivation in batch, fed-batch, fixed bed and perfusion processes. We have also detailed the historical background of insect

cell culture, the size and direction of the market, the future applications of insect cells, the benefits and the drawbacks of the BEVS as an important biotechnological platform.

The original contributions of the present work have been developed along two axes. The first axis concerns the study of the physiology of High-Five cells and of the environmental conditions affecting their growth and death (Chapters 2 to 3), followed by a comprehensive study of the metabolism of High-Five cells (Chapter 4). The second axis addresses the elaboration of fed-batch and fixed bed processes (Chapters 5 and 6) using High-Five cells based on their specific physiology and metabolism.

We have chosen to work in parallel with the Sf-9 and High-Five cell lines to compare our new results on High-Five cells to the available literature on Sf-9 cells. Both cell lines were subcultivated in shake-flasks, adapted and cultivated in our own serum- and protein-free YPR medium (Ikonomou et al., 2000) to which dextran was added (Chapter 2). Bioreactor studies were carried out in a Celligen-Plus™ bioreactor (New Brunswick Scientific, Edison, NJ).

We have chosen to use the *Autographa californica* (AcMNPV) baculovirus, because it is the most commonly used and widely referenced baculovirus. We used wild-type virus and two recombinant virus constructs coding for the *E. coli* β-galactosidase (β-gal) and the human secreted alkaline phosphatase (seAP) genes under the control of the polyhedrin promoter.

A lot of fundamental work on IC-BEVS in the literature employs this AcMNPV coding for the β-gal gene to express a model cytosolic protein. This β-gal is an enzyme that removes, by hydrolysis, the terminal β-D-galactose residues in β-galactosides. It is a non-glycosylated cytosolic protein of 1023 amino acid length (116 kDa). It is released into the supernatant at the end of the infection cycle when cells are lysed and it is susceptible to proteolytic cleavage.

An alternative recombinant form of the same AcNMPV baculovirus encoding the seAP gene was used as model in the IC-BEVS to express a secreted glycoprotein in accordance with numerous recent publications. The alkaline phosphatase is a membrane-bound glycoprotein that catalyzes the hydrolysis of orthophosphoric monoesters to an alcohol and orthophosphate. It is distributed in various organs. Placental human alkaline phosphatase (PLAP), a 535 amino acid length (68 kDa MW) protein, has two putative glycosylation sites (Asn^{122} and Asn^{249}) but only the Asn^{249} is glycosylated.

In the present work, seAP is a truncated form of PLAP inserted in a baculovirus vector by Davis et al. in 1992.

The work presented in Chapter 2 is the development of a medium able to prevent the formation of a cell ring on shake-flask and bioreactor vessel walls. This cell ring, mainly composed of dead cells, is deleterious for the cultivation. It contributes to a decrease in the number of free cells and diminishes the overall viability. In this study, we have observed that in serum-free media, the presence of an optimal type (67 kDa) and concentration (1 g/l) of dextran decreases this cell ring. Therefore, when adapted and cultivated into media supplemented with dextran, High-five cells are bigger in its presence, are able to reach ca 9-10x10^6 cells/ml with a high growth rate (μ = 0.025 h^{-1}) and a high volumetric productivity. This phenomenon probably results from the adsorption of dextran simultaneously on the recipient's inner surfaces and on cell membranes, which inhibits the complex cumulative and indirect effect of adsorption of cells on the vessel surfaces. Hence, the use of 1 g/l of 67 kDa dextran as a supplement to the YPR medium was kept for the rest of this work.

In Chapter 3, we present the environmental conditions affecting insect cell death. We firstly developed and compared the methods able to distinguish between apoptosis and necrosis in insect cells. Secondly, we used these methods to understand what conditions cause cell death in High-Five cultures. Among the various moderate stresses tested, physical and chemical stresses (increase of shear stress, absence of Pluronic® F-68, etc.) triggered a shortening of the growth phase and caused cell death by necrosis, but did not really seem to affect apoptosis. On the contrary, moderate metabolic stresses such as the removal of hydrolysates such as Yeastolate and Primatone®, the deprivation of glucose, the presence of the by-product ammonia, all seemed to induce apoptosis. However, the presence of the by-product lactate did not seem to cause apoptosis of High-Five cells. We were able to show that the antioxidant activities of Yeastolate and Primatone could explain why their absence caused programmed cell death.

Chapter 4 is the comprehensive study of High-Five cell metabolism using a metabolic flux network. Prior published information on insect cell metabolism was used to build the metabolic pathway network operating in these cells. Extracellular consumption and production of metabolites were directly used in the flux network as catabolic pathways. The biomass composition of the cells was used jointly with

literature data to determine the anabolic intracellular fluxes (e.g. fluxes of consumption of amino acids for anabolism were deduced from the specific protein production rate and the occurrence of each amino acid in insect proteins). The global pathway maps of High-Five and Sf-9 cells were elaborated under various environmental conditions (growth, infection, depletion of glucose and presence of by-products).

The use of this metabolic flux network revealed differences in the central metabolism between High-Five and Sf-9 cells: High-Five cells seem to consume preferentially Asn whereas Sf-9 prefer Gln. High-Five cells are able to consume lactic acid and Ala when the glucose becomes deficient. Moreover, High-Five cells do not consume the same amino acids as energy source as Sf-9 cells. Finally, the pathway of Asn use as energy source by High-Five suggested by the metabolic flux network was proven experimentally using radioactively labeled Asn^{14}C.

The two last chapters (Chapters 5 and 6) are the application of the knowledge gained throughout the previous chapters to develop fed-batch and fixed bed processes. We did not develop microcarriers or perfusion culture using the High-Five cell line in YPR media because these aspects have already been studied (Ikonomou, 2002; Ikonomou et al., 2002).

Chapter 5 is the development of a fed-batch strategy specific to High-Five cells. This feeding strategy, consisting of different pulses, was developed to increase the productivity of cells during infection (based on the metabolic observations made in Chapters 3 and 4). Our approach was based on an understanding of the cells' needs during infection and could be summarized in the following questions: what nutrients are needed to be brought in as additive to the cells? when do the cells need it(them)? at what level (how much)? how often? and at what concentration? Consequently, we developed a High-Five fed-batch strategy consisting in a pulsed supplement composed of glucose, glutamine, Primatone and yeast extract diluted in basal medium whose formulations evolve as a function of cellular requirements. This feeding strategy sustains a recombinant protein production of β-gal and seAP 5.5 fold higher than the one corresponding to batch infections without medium changeover. Moreover, the above fed-batch supplementation allows to obtain slightly higher volumetric productivities compared to a total replacement of the growth medium by fresh medium upon infection without the use of an expensive and constraining cell retention system.

Chapter 6 is the application of the previously gained knowledge to elaborate a fixed bed reactor system with High-Five cells for recombinant protein production. In this work, we investigated glass fibres (Vel: VWR International, Leuven, Belgium) and Dacron® PET (DuPont de Nemours, Paris, France), Fibra-Cel® (New Brunswick Scientific, Edison, NJ) and BioNOC II™ (Cesco Bioengineering, Hsin-Chu, Taiwan) macroporous microcarriers as supports. We also investigated the culture in the BelloCell™ system, a new disposable fixed bed reactor from Cesco Bioengineering. We found that cells cultivated in a fixed bed produced more by-products and wasted more nutrients in comparison to a free cell (suspension) control. Besides, we attempted to find what are the key controlling parameters for a packed bed bioreactor with High-Five cells. Based on the metabolism of cells in a packed bed, we have developed a packed bed bioreactor strategy based on BioNOC II microcarriers that allows to sustain a density of 400.10^6 cells/g of support and a volumetric protein production level 2.5 fold higher that in batch.

Finally, we have summarized the new knowledge brought by this work on High-Five cell metabolism and physiology, and on the advances in the elaboration of fed-batch and fixed bed processes using these cells in a final chapter with general conclusions and perspectives for continuation of research.

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PART I: INSECT CELL CULTIVATION

1

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Chapter 1 : Literature review

1.1. HISTORICAL BACKGROUND

Contrary to the general view, the culture of invertebrate cells in vitro is not recent. It began in 1915 when the German entomologist Richard B. Goldschmidt worked with the silkmoth (Goldschmidt, 1915). He noticed in vitro spermatogenesis and maintained cells alive in hemolymph from homologous insect as culture medium. Between 1917 and 1922, Stanley Glaser made efforts to develop insect tissue culture. In 1935, William Träger achieved the first long term cultivation of insect cells (a culture of silkworm ovary cells maintained for 3 weeks in a medium consisting of hemolymph, inorganic salts, maltose and digested egg albumin).

In 1956, Gerard R. Wyatt isolated cells from the silkworm *Bombyx mori* and analyzed the amino acids, organic acids, organic salts and sugars present in the hemolymph. He maintained ovarian cells alive for 2 weeks (Maramorosch, 1999). In 1962, Thomas Grace isolated the first four lepidopteran insect cell lines and developed the first synthetic growth medium, already in use today, for the cultivation of insect cells. This medium required serum as additive. On that same year, the first International Colloquium on Invertebrate Tissue Culture was organized in Montpellier in France. Today, this meeting is again organized every couple of years and brings together scientists and people from industry from the entire world. Two years later, Grace established the first mosquito cell line (Maramorosch, 1999). In the later 60's, other cell lines were also developed, most of them lepidopteran.

In the same period, wild-type baculoviruses infecting in vivo lepidopteran larvae came under intensive study. Originally propagated in larvae, they were applied to the in vitro production of bioinsecticides. Although, in fact, the first baculovirus application in agriculture as a bioinsecticide was in1892, the first extensive trials of baculovirus efficacy and safety were carried out in 1975 by the US Environmental Protection Agency (EPA) (Maeda, 1995). In the 70's and 80's, the number of actors working in insect cell cultivation increased and the interest of invertebrate cell cultivation surpassed that of classical entomological studies. The high contribution of Karl Maramorosch was that he adapted insect cell cultivation to a large scale, thanks to the development of universal insect cell media, both with (medium MM, Mitsuhashi and Maramorosch 1964) and without serum (medium MM-SF, Mitsuhashi 1982).

In the beginning of the 80's, several scientist (Wilkie et al., 1980; Weiss et al., 1981) developed the first serum-free media supporting large scale cultivation by replacing serum by sterols and lipids. Their main motivations were to reduce the cost and the

lot-to-lot variation, while today, the use of serum-free media is essentially dictated by safety considerations (Galbraith, 2002).

In 1983, Smith et al. (1983) constructed the first recombinant baculovirus to produce recombinant human interferon. The use of this Baculovirus Expression Vector System (BEVS) with co-cultivated Insect Cells (IC) opened a whole new field of activity in the area of protein expression, and the initial interest on biopecticides using wild-type virus was expanded by modifying the baculovirus genome. In 1988, the first recombinant baculovirus expressing an insect-specific neurotoxin was developed to increase its biopesticide action (Carbonell et al., 1988). In 1991, Hink et al. tested 3 different recombinant baculoviruses for protein expression in 23 insect cell lines (Hink et al., 1991). Since then, the genome of *Autographa californica* (AcMNPV), the most widely used baculovirus, has been completely sequenced (Ayres et al., 1994).

As we enter the 21st century, new fields of baculoviruses applications are opening up, such as Baculovirus Display (Ojala et al., 2001) and the use of baculoviruses as vectors for gene delivery (Condreay and Kost, 2003; Kost et al., 2005). Today, baculoviruses as bioinsecticides are an efficient, cost-effective substitute to chemical pesticides (Henrnandez-Crespo et al., 2001). Genetically modified baculoviruses constitute a powerful tool for the expression of glycosylated recombinant proteins (r-proteins), especially in the research and medical area (Rhodes, 1996). They are used either as part of the IC-BEVS technology platform or with mammalian cells as transfer vectors (Hu, 2005).

The first therapeutic manufactured in IC-BEVS, a veterinary vaccine, was approved and commercialized in 2000 (Intervet International, The Netherlands) (Walsh, 2005). Interest in Insect-Cell technology shows a strong and consistent growth, as seen by the fact that today 3 future human vaccines produced by IC-BEVS are in clinical phase III trials (EMEA: European Medicines Agency).

1.2. INSECT CELL PHYSIOLOGY

Since Thomas Grace established the first insect cell lines from *Antheraea eucalypti* in Australia in 1962, ca. 500 such lines have been isolated from more than 100 insect species encompassing more than 6 orders (Lynn, 2001). Among them, some lines are used for entomological studies, while cell lines from the dipteran *Drosophila melanogaster* (whose genome is now sequenced) are mainly used for genetic studies and for constitutive gene expression (Park et al., 1999). Lepidopteran cell lines are mainly used with the BEVS for the expression of r-proteins and for the production of baculovirus bioinsecticides, particularly lines from *Bombyx mori* (*B.mori:* silkworm), *Mamestra brassicae*, *Spodoptera*

frugiperda (*S. frugiperda*: "fall army worm") and *Trichoplusia ni* (*T.ni*: "cabbage looper") (O'Reilly et al., 1992). Among them, Sf-9, Sf-21, Tn-368 and BTI-TN-5B1-4 isolated from *S. frugiperda* and *T. ni* (nocturnal butterflies) are the cell lines most widely used in industrial applications.

The first line to be intensively used in research and technological applications was Sf-21, an ovarian cell line from *S. frugiperda* established by Vaughn and co-workers in 1977. The Sf-9 cell line was derived from Sf-21 (Smith et al., 1983). Thanks to, its improved growth and baculovirus infection characteristics, it has progressively replaced Sf-21 in the research and production fields, to the extent that today it represents perhaps the most widely used of all insect cell lines. Tn-368, was derived from ovarian tissues of *T. ni* (Hink, 1970). However, this cell line does not appear lately in the scientific literature (Ikonomou et al., 2003).

BTI-TN-5B1-4 (with sometimes different spelling) cells are a clone of the embryonic Tn-5 cell line isolated from *T. ni* (Hink, 1970). More specifically, the BTI-TN-5B1-4 cell line represents a clone of the *T. ni* egg cell line BTI-TN-5B1-28 (Granados et al., 1994). Robert Granados, of the Boyce Thompson Institute at Cornell University (Ithaca, NY) patented this cell line in 1994 (Granados, 1994) and it was subsequently commercialized by Invitrogen (Carlsbad, CA) under the name of "High-Five™ cells", thanks to its capacity of reaching higher cell densities with higher growth rate and higher production rate (for many proteins) than the classical Tn-5. The High-Five cell line showed from the beginning a superior capacity for secreted glycoprotein production compared to Sf cell lines (Davis et al., 1993; Saarinen et al., 1999). Some key characteristics of the Sf-9 and High-Five cell lines are summarized in Table 1-1.

The Sf lines are adapted to suspension cultivation and are easily detached from cultivation surfaces by gentle agitation without trypsinization (O'Reilly et al., 1992). Tn lines were originally anchorage-dependent, but today they are well adapted to suspension cultivation. Moreover, upon microcarrier cultivation, High-Five cells are more easily detached than Sf-9 cells (Ikonomou et al., 2002). Among Sf lines, Sf-21 are more fragile than Sf-9, less tolerant to osmotic, pH and shear stress than Sf-9, and have lower growth rate (O'Reilly et al., 1992). Today, the use of Sf-21 has diminished to the benefit of Sf-9 cells. The latter are able to better amplify the baculovirus (Wang et al., 1992), while High-Five is the insect cell line that produce more r-protein, up to 10-20 fold more compared to Sf-9 (function of the r-protein produced) (Palomares and Ramirez, 1998; Saarinen et al., 1999). High-Five cells are more robust to shear stress and osmotic shocks than Sf-9 (Kioukia et al., 1995), although Sf-9 is more resistant to thermal shock (Gerbal et

al., 2000). High-Five cells (15 µm) are bigger with higher protein content than Sf-9 cells (13 µm), and their cell size distribution is wider than Sf-9 cells (This thesis, Chapters 2 and 4). However, the cell size depends on medium osmolarity, shear stress, cell state (viable, apoptotic, etc.) (Palomares et al., 2001).

Table 1-1: Summary of difference between High-Five and Sf-9 cells.

		Sf-9 cells	Both cell lines	High-Five cells
Physiology – culture conditions				
Cell Size		13 µm		Bigger (15 µm)
Reached cell density (10^6cells/ml) in batch			4-10	
Growth rate		0.025 h^{-1}		Higher (0.030 h^{-1})
Optimal growth temperature			27°C	
Optimal protein production temperature			25-29°C	
Optimal pH		6.2-6.4		6.2-6.3
Metabolism				
Growth	Glucose consumption	+++		Higher (++++)
	Glutamine consumption		++	
	Asparagine consumption	+		Higher (++)
	Amino acid consumption	++		Higher (+++)
	Oxygen consumption		++++	
	Lactate production	+		Higher (++)
	Ammonia production	++		Higher (+++)
	Alanine production		++	
Infection	Glucose consumption	++		Higher (++++)
	Glutamine consumption	+		Higher (+++)
	Asparagine consumption	-		Higher (++)
	Amino acids consumption	+		Higher (+++)
	Oxygen consumption	++++		Higher (+++++)
	Lactate production	-		Higher (++)
	Ammonia production	+		Higher (++)
	Alanine production		++	
Sensitivity	Thermal shocks	Less sensitive		More sensitive
	Changes of pH		Weak sensitivity	
	Osmotic shocks	More sensitive		Less sensitive
	Shear stresses	More sensitive		Less sensitive
	Changes of DO		Moderate sensitivity	
	Ammonia by-product	More sensitive		Less sensitive
	Lactate by-product	More sensitive		Less sensitive
Production				
Yield of r-protein		Good		Best (up to 10 times more)
Yield of virus		Best		Good
Post-translational modification			Complex glycosylation and sialylation	
Secretion of r-protein		Good		Best
Industrialisation				
Adapted to serum-free media			Yes	
Availability of adapted media		Best		Good
Scaling-up possibilities		Good		Best
Established knowledge on				
Cell line characterization			Good	
Metabolism of cells		Good		Moderate
Culture conditions		Very good		Good
Process development (fed-batch, perfusion, fixed-bed, etc.)		Good		Moderate

-: almost null, + : weak, ++ : moderate, +++ : high, ++++, higher, +++++: very high

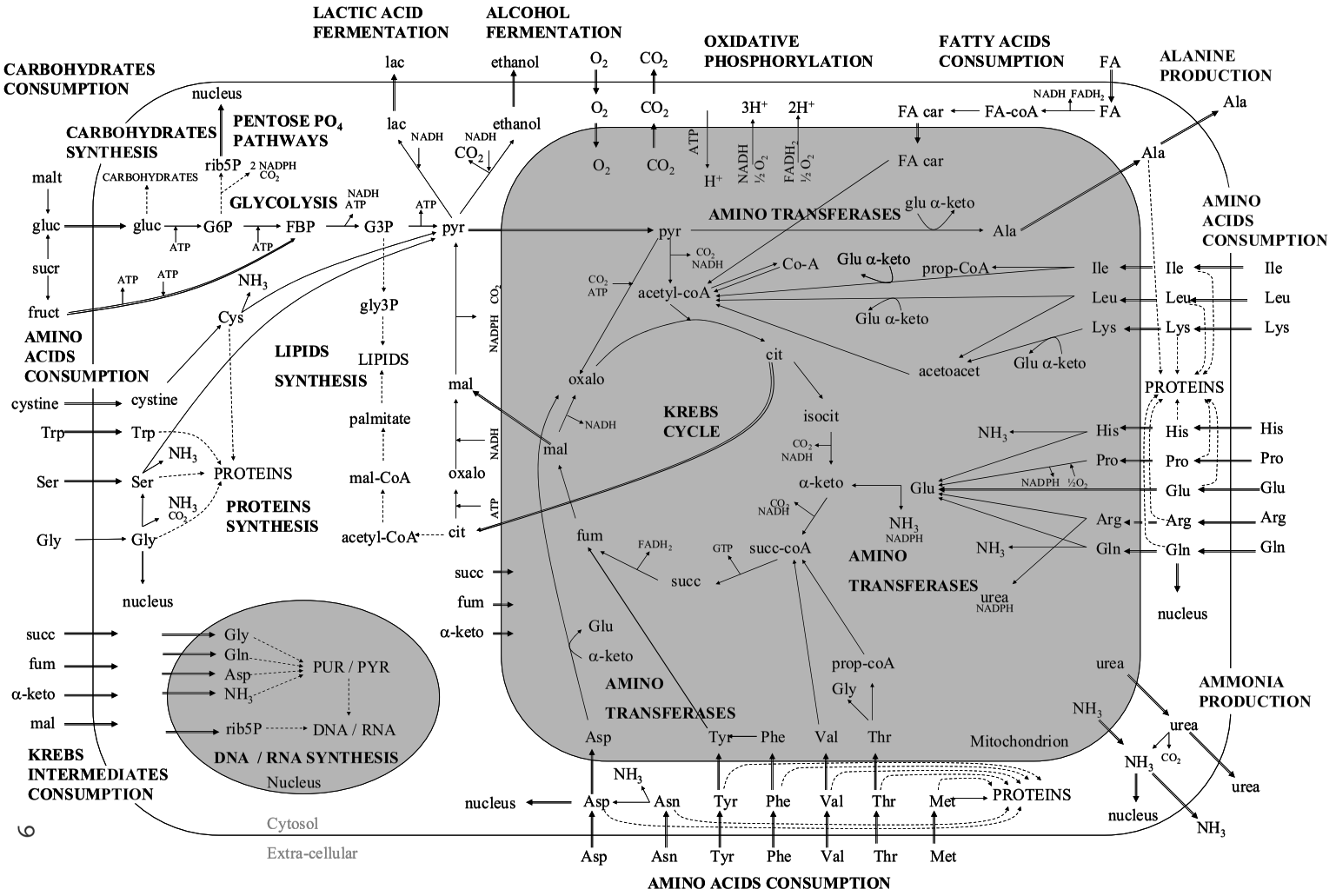
In any case, when infected, Sf and Tn cells are bigger (Lloyd et al., 2000). The maximal reported cell densities obtained in serum-free batch culture with High-Five was $9.6x10^6$ cells/ml cultivated in YPR medium (This thesis, Chapter 2) and was $8.1x10^6$ cells/ml with Sf-9 cells (Rhiel et al., 1997) cultivated in Sf900-II. Insect cell lines have polyploid chromosomes, and changes in this polyploidy could occur during the cultivation (Léry et al., 1999; Doverskog et al., 2000). These cell lines are able to grow during long term passaging, during which, however, morphological and physiological changes may occur: decrease of productivity and increase of growth rate and cell diameter (Donaldson and Shuler, 1998b) and lower susceptibility to growth enhancement by conditioned medium (Calles et al., 2006a; Calles et al., 2006b).

Finally, the tendency of baculovirus infected cultured insect cells towards production of defective interfering particles ("passage effect") increases with increased cell passage number (Kool et al., 1991; Wickham et al., 1991; Pijlman et al., 2001). It is recommended to renew the working subcultivation after 30 passages (Palomares et al., 2006).

1.3. INSECT CELL METABOLISM

The knowledge of insect cell lines is primordial for the elaboration of new media and for designing feeding strategies. Several works on lepidopteran cell metabolism have appeared in the last decade. Most of them treat the metabolism of Sf-9 cells and only a few that of High-Five cells. Insect cell metabolism has some notable differences from that of mammalian cells in culture. The metabolic activity of High-Five cells is more intense (nutrient uptake rate 1.4 to 2.5 times faster) in comparison to Sf-9 cells (Table 1-2). The latter have a lower specific rate of consumption of asparagine (Asn) and aspartic acid (Asp) than High-Five (Yang et al., 1996), and have a lower rate of production of lactate (Bédard et al., 1993). Besides, their metabolic regulation could be different.

Figure 1-1 (next page): Metabolic pathways of High-Five cell lines
Full lines, dashed lines and double lines represent, respectively, the catabolic pathways, the anabolic pathways and the transfer of compounds from one cellular compartment to another one. α –keto: α –ketoglutarate, acetoacet: acetoacetate, cit: citrate, FA: fatty acid, FA-car: fatty acid carnitine, FA-CoA: fatty acid-CoA, FBP: fructose-1,6-bisphosphate, fruct: fructose, fum: fumarate, G3P: 3-phosphoglycerate, G6P: glucose-6-phosphate, gluc: glucose, gly3P: glycerol-3-phosphate, icocit: isocitrate, lac: lactate, mal: malate, mal-CoA: malonyl-CoA, malt: matose, oxalo: oxaloacetate, prop-CoA: propionyl-CoA, PUR: purines, PYR: pyrimidines, pyr: pyruvate, rib5P: ribose-5-phosphate, succ: succinate, succ-CoA: succinyl-CoA, sucr: sucrose


LACTIC ACID FERMENTATION
ALCOHOL FERMENTATION
OXIDATIVE PHOSPHORYLATION
FATTY ACIDS CONSUMPTION
ALANINE PRODUCTION
CARBOHYDRATES CONSUMPTION
CARBOHYDRATES SYNTHESIS
PENTOSE PO4 PATHWAYS
GLYCOLYSIS
AMINO TRANSFERASES
AMINO ACIDS CONSUMPTION
LIPIDS SYNTHESIS
KREBS CYCLE
PROTEINS SYNTHESIS
AMINO TRANSFERASES
PROTEINS
AMMONIA PRODUCTION
KREBS INTERMEDIATES CONSUMPTION
DNA / RNA SYNTHESIS
Nucleus
Mitochondrion
Cytosol
Extra-cellular
AMINO ACIDS CONSUMPTION
malt
gluc
sucr
fruct
G6P
FBP
G3P
pyr
lac
ethanol
rib5P
gly3P
palmitate
mal-CoA
acetyl-CoA
cit
isocit
oxalo
mal
fum
succ
α-keto
succ-coA
Co-A
prop-CoA
acetoacet
FA
FA car
FA-coA
Ala
Cys
cystine
Trp
Ser
Gly
His
Pro
Glu
Arg
Gln
Ile
Leu
Lys
Asp
Asn
Tyr
Phe
Val
Thr
Met
urea
PUR / PYR
DNA / RNA

1.3.1. CARBOHYDRATE METABOLISM

Glucose is the most important carbohydrate for insect cells (Figure 1-1). It is identified as the preferred energy and carbon source (Drews et al., 1995). Insect cells are known to have a higher specific rate of consumption of glucose than mammalian cells in culture (CHO, BHK, etc.). Like mammalian cells, insect cells are able to grow in media containing only glucose as carbohydrate (Landureau, 1969; Reuveny et al., 1992). However, Sf-9 and High-Five are able to consume others carbohydrates as carbon source such as fructose and maltose, following the hydrolysis of this dimeric sugar outside the cell (Reuveny et al., 1992; Bédard et al., 1993). But fructose was only consumed after glucose depletion (Ikonomou et al., 2003). Sucrose, which is added to adjust osmolarity, is neither consumed in growth phase by Sf-9 cells (Reuveny et al., 1992; Bédard et al., 1993; Drews et al., 1995) nor by High-Five cells (Rhiel et al., 1997) and is only consumed by Sf-9 cells during baculovirus infection (enzyme not identified) (Wang et al., 1993a; Sugiura and Amann, 1996). Trehalose that is the major carbohydrate in hemolymph, was found to be not consumed if other carbon sources are present (Vaughn, 1973).

Carbohydrate metabolism is regulated in a similar way as in other eukaryotic cells. An analysis of the metabolic flows of insect cells has shown that these cells possess a fully functional TCA cycle (Bhatia et al., 1997).

Under conditions of lack of glucose, the growth of Sf-9 and High-Five cells were observed to stop (Bédard et al., 1993; Drews et al., 2000), but cells can stay alive if enough quantities of amino acids are present. Besides, the absence of glucose triggers apoptosis in Sf-9 cells (This thesis, Chapter 3), whereas High-Five cells can still stay alive by consuming lactate (Drugmand et al., 2005). On the contrary, excessive amounts of glucose result in the accumulation of lactate and alanine as by-products (Bédard et al., 1993; Öhman et al., 1995; Mendonça et al., 1999).

Concerning the Krebs cycle intermediates that are present in some insect cell media, they can directly feed the Krebs cycle and be used as carbon source. But, their presence in media seems useful only in the lag phase. Sf-9 and High-Five cells seem not to consume them during growth phase (Schlaeger, 1996a; This thesis, Chapter 4), and their absence in TC-100 medium has no effect on cell growth (Gardiner and Stockdale, 1975).

Table 1-2: Metabolic coefficients (µmol/10^9cells.h) for High-Five and Sf-9 cells in growth phase cultivated in serum-free media under no nutrient limiting conditions

Metabolic rates (µmol / 10^9 cells.h)	Growth phase		Infection phase	
	High-Five	**Sf-9**	**High-Five**	**Sf-9**
Glucose consumption	100-200[a,f,d,g,h]	51-93[a,f,d,g,h]	160-260[a,d,e,h]	59-61[d,h]
Glutamine consumption	34-54[d,f,g,h]	23-40[d,f,g,h]	58-78[a,d,e,h]	18-19[d,h]
Oxygen consumption	200-460[c,d,g, h]	280-650[c,d,g,h]	320-1220[a,b,h]	200-760[a,b,h]
Lactate production	6-25[a,d,h]	4-12[a,g,h]	14-39[d,h]	3[h]
Ammonia production	55-180[d,g,h]	2-18[g,h]	38-46[d,h]	22[h]
Alanine production	32-54[d,g,h]	39-45[d,g,h]	21-50[d,h]	27-28[d,h]

a: Bédart et al., 1993; b: Kamen et al., 1996; c: Palomares and Ramirez, 1996; d: Rhiel et al., 1997; e:Donaldson and Shuler, 1998 ; f :Mendoça et al.,1999 ; g: Benslimane et al., 2005 ; h :This thesis, Chapter 4

1.3.2. AMINO ACIDS

Insect cells can utilise amino acids for biosynthesis of protein and DNA/RNA and energy. Glutamine (Gln) is known as the main amino acid used as energy source in Sf cultures (Rhiel et al., 1997; Drews et al., 2000). The pathway affecting Gln in Sf-9 cells is highly regulated and involves four enzymes (Figures 1-1 and 1-2): glutaminase, glutamate dehydrogenase, glutamine synthetase and alanine aminotransferase (Öhman et al., 1996). When glucose is limiting (<7 mM), Gln is converted by glutaminase to glutamic acid (Glu) thereby producing ammonia. After that, Glu is converted to α–ketoglutarate by glutamate dehydrogenase and feeds the Krebs cycle (Wang et al., 1993a; Drews et al., 2000). (Öhman et al., 1996). This pathway is reversible because the lepidopteran cells possess a glutamine synthetase (Drews et al., 2000), and this could be used by Sf-9 cells, but not by High-Five cells, to synthesize Gln if ammonia is available (Öhman et al., 1996). However, the fact that the growth is affected by the absence of Glu, proves that this pathway of Glu synthesis is not completely efficient (Öhman et al., 1996; Mendonça et al., 1999). Under excess of glucose, insect cells have a safe way to dispose of nitrogen by-products in the form of alanine. Specifically, Sf-9 cells utilise alanine aminotransferase, instead of glutamate dehydrogenase, to convert Glu to α–ketoglutarate by producing alanine, instead of ammonia (Öhman et al., 1995).

Asn is consumed extensively by High-Five cells in culture and by Sf-9 cells under conditions of Gln limitation. Asn used up quickly by High-Five cells (Danner et al., 1995; Yang et al., 1996; Rhiel et al., 1997) and its depletion seems to coincide with the onset of the stationary phase (Yang et al., 1996).

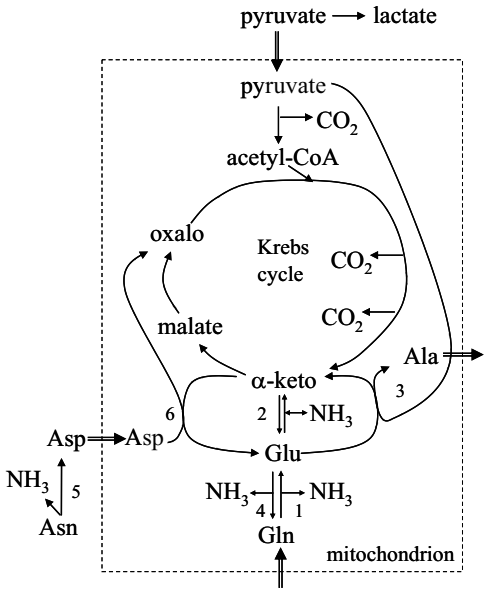


Figure 1-2: Gln/Glu and Asn/Asp metabolism in insect cells.
Enzymes: 1 glutaminase; 2, glutamate dehydrogenase; 3, alanine aminotransferase; 4, glutamine synthetase ; 5, asparaginase ; 6 aspartate aminotransferase.

However, supplementation of Asn in High-Five cell culture, does not seem to extend the growth phase, but instead to increase its consumption rate (This thesis, Chapter 4). Its consumption by High-Five cells is highly regulated and involves enzymes shared between the Gln/Glu and Asn/Asp metabolism (This thesis, Chapter 4).

The rest of the amino acids are consumed to a lesser extend and differs from one cell line to the other. According to Drew et al. (1995), arginine (Arg), Asn, Asp, Gln, Glu, methionine (Met) and serine (Ser) are used by Sf-9 for energy production, whereas Arg, Asp, cystine (Cys), Glu, histidine (His), leucine (Leu), lysine (Lys), Met, threonine (Thr) could be used as energy source by High-Five cells (This thesis, Chapter 4).

The majority of the amino acids are not synthesized by insect cells (Bhatia et al., 1997) and the lack of just one of them can stop the growth. According Landureau and Jollès (1969), Arg, Cys, His, Met, phenylalanine (Phe), proline (Pro), valine (Val), tryptophan (Trp), and tyrosine (Tyr) are crucial to sustain the growth of EPa insect line form *Periplaneta americana* (Landureau and Jollès, 1969), whereas Ferrance et al. (1993) and Radford et al. (1997) found that isoleucine (Ile), Lys, His, Leu and Thr are also crutial for Sf-9 cells. Moreover, Cys and glycine (Gly) are essential for Sf-9 cells that do not express methylenetetrahydrofolase (Tremblay et al., 1992). The presence of Met and Tyr is necessary to delay cell death in Sf-9 cultivation (Mendonça et al., 1999).

Some amino acids (Arg, Asn, Asp, Gln, Glu, Gly and Ser) are required to sustain high growth rate with Sf-9 (Ferrance et al., 1993; Rhiel et al., 1997) and High-Five cells (Wang et al., 1993b; Rhiel et al., 1997). Radford et al. (1997) have shown the same amino acids (Arg, Asn, Asp, Gln, Glu, Gly, Ser) are also required during the infection phase to obtain high protein production rates.

1.3.3. By-products

Insect cell cultures usually accumulate low amounts of lactate. This acid is a product of glucose metabolism in eukaryotic cells (Figure 1-1). Sf-9 cells do not produce lactate under oxygen-sufficient conditions even in media with high glucose content (>40 mM). In contrast, High-Five cell cultures accumulate relatively high amounts of lactate, usually between 10 to 20 mM (Sugiura and Amann, 1996; Yang et al., 1996).

But if these cells are cultivated in a non adapted medium (e.g. Sf900-II), accumulation of lactate could reach 45 mM during growth phase and up to 30 mM during infection (Rhiel et al., 1997). The lactate produced can be consumed by both High-Five (Drugmand et al., 2005) and Sf-9 cells (Kamen et al., 1991) after carbohydrate depletion. The toxicity of lactate to insect cells is probably similar than to mammalian cells (BHK) due to the medium acidification and the increase of osmolarity (Cruz et al., 2000). Moreover, the export of lactate out of the cells triggers a decrease of the extracellular pH. This toxic effect of lactate up to 10 mM seems not to affect the growth phase of Sf-9 cells (Palomares and Ramirez, 1996) while 8 mM of lactate inhibit the growth of Bm-5 cells (Stavroulakis et al., 1991). Lactate accumulation up to 12.5 mM does not trigger necrosis of Sf-9 and High-Five cells, but seems to slightly increase apoptosis (This thesis, Chapter 3).

In the presence of lactate, High-Five cells cultivated in single-cell suspension may produce clumps and the extent of their growth phase may decrease (Ikonomou et al., 2001). Moreover, High-Five cells seem to produce lactate under stress conditions (lack of glucose, shear stress, etc.), and its detection in a given culture is an indicator of such stress but also the presence of lactate seems to provoke a further increase of stress on the cells by a kind of positive feed-back loop (Drugmand et al., 2005). At the end of a batch culture, after glucose exhaustion from the medium, High-Five cells are able to use this accumulated lactate as carbon source.

Ethanol, another by-product of glucose metabolism (Figure 1-1), is usually not produced by mammalian cells, while insect cells produce it. Its production is usually almost insignificant (This thesis, Chapter 4). Nevertheless, (Drews et al., 2000) has reported a production of 6 mM ethanol in serum-free culture of Sf-9 cells (pathway not

clearly identified). Ethanol was also produced to a maximum of 11.5 mM from three insect cells lines cultivated in the Mitsuhashi-Maramorosch (MM) medium (Takahashi et al., 1995).

Ammonia and alanine are other important by-products produced from the catabolism of amino acids (Figures 1-1 and 1-2). The toxicity of ammonia on insect cells is not clearly identified, but probably results, like in mammalian cells, from the acidification of the mitochondria and cytoplasm (due to the equilibrium $H_2O + NH_3 \leftrightarrow NH_4^+ + OH^-$) and the modifications of K^+ gradient due to the competition of K^+ with the ammonium cation for the Na^+/K^+-ATPase transporters (Martinelle et al., 1996; Schneider et al., 1996). Besides, the export of ammonia out of the cells triggers an increase of the extracellular pH. Contrary to mammalian cells, the specific production rate of ammonia by insect cells is higher but insect cells are less sensitive to its toxicity (Bédard et al., 1993; Schneider et al., 1996). Sf and Bm cell lines, under optimal culture conditions, produce less ammonia during growth phase than Tn cells (Bédard et al., 1993; Öhman et al., 1996).

Sf-9 growth is not affected if ammonia reaches 10 mM (Bédard et al., 1993). Moreover, Sf-9 cells are able to use ammonia if present in the culture medium to produce Glu from α–ketoglutarate (Bédard et al., 1993; Öhman et al., 1995; Palomares et al., 2006). As mentioned above, under glucose excess, Sf-9 cells produce alanine instead of ammonia. This amino acid by-product is not toxic for the cells at concentrations up to 100 mM (Öhman et al., 1995) and serves as an ammonia sink to detoxify the medium. On contrary, High-Five cells produced up to 20 mM ammonia during the growth phase from Gln and Asn metabolism (Sugiura and Amann, 1996; Yang et al., 1996; Chico and Jäger, 2000). Ammonia production depends on the initial Gln and Asn concentration in the culture medium (Rhiel et al., 1997). The growth of High-Five cells is not affected by addition of ammonium salt up to 10 mM but 30 mM have been found to affect slightly the growth but severely the productivity of r-protein (Yang et al., 1996). Moreover, 25 mM of ammonia trigger apoptosis in High-Five cells (This thesis, Chapter 3).

1.3.4. LIPIDS

Insect cells have incomplete lipid metabolism and need lipid supplementation. They have a limited capacity of synthesising, desaturating and elongating fatty acids (Öhman et al., 1995). Besides, these cells cannot synthesize cholesterol, which is required for the formation of hormones and membranes (Mitsuhashi, 1989). However, insect cells are able to use lipids for the production of energy (Luukkonen et al., 1973). Lipid deprivation in the medium causes production of deficient baculovirus and cell degeneration (Goodwin, 1991).

1.4. THE BACULOVIRUSES

1.4.1. MORPHOLOGY

Viral infections are common in insects. About 1600 viruses grouped into more than 10 families on the basis of their morphology have been reported to cause diseases in more than 1100 insect species (Glazer and Nikaido, 1995). One of these families, the Baculoviridae containing at least 600 species of viruses, is restricted in host range to a small number of invertebrate species. The name baculovirus refers to the rod-shaped capsid of the virus particle, which measures 40-50 nm in diameter and 200-400 nm in length (O'Reilly et al., 1992). Baculoviruses are double stranded, circular and helicoidal DNA viruses with a genome size of 80 to 230 kb.

The Baculoviridae family includes three types of lytic viruses: Granulovirus (GNV), Nucleopolyhedrovirus (NPV) and Nonoccluded virus (NOV), which are classified on the basis of their envelope (Winstanley and O'Reilly, 1999). The GNV viruses have only one virion occluded in granular crystalline matrix, whereas NPV present multiple virions embedded into a occlusion body (also named polyhedra) made of a polyhedral crystalline matrix composed of the protein polyhedrin enveloped by a carbohydrate coat named calyx (Glazer and Nikaido, 1995; Winstanley and O'Reilly, 1999). NPV viruses are classified as multiple (MNPV) or single (SNPV) Nucleopolyhedrosis viruses depending of the number of viruses into the capsid. The MNPV polyhedra measure between 0.8 and 5 μm and could contain more than 100 viruses. The NPV virus are the only viruses known to have two phenotypes: budded virus (BV) and occluded virus (OV) to complete their life cycle in nature (Glazer and Nikaido, 1995). The polyhedra of OV protect them from adverse environment conditions. Both virus types have the same nucleocapsid surrounding the DNA-containing core but only OV possessed the crystalline matrix embedded virus, while BV virus are only constituted of one nucleocapsid. The NOV viruses constitute a rare type of non occluded virus (Luckow and Summers, 1988; O'Reilly et al., 1992).

The best studied viruses are the *Bombyx mori* SNPV (BmNPV) and the *Autographa californica* MNPV (AcMNPV). The wildtype BmNPV host range is limited to *B. mori* larvae and that of AcMNPV *S. frugiperda, T. ni* and *Orgyia pseudotsugata* larvae. The entire DNA sequence of AcMNPV is available (Glazer and Nikaido, 1995; Volkman, 1997). It is the most widely used virus as vector for r-protein expression due to its capacity to infect in vitro several lepidopteran cell lines.

1.4.2. LIFE CYCLE

The life cycle of wildtype NPV viruses is temporally divided in four phases (Figure 1-3): immediate early (α), delayed early (β), late (γ) and very late (δ) phase. The infection begins by the ingestion by larvae of the occluded virus present in the environment. The polyhedrin matrix is dissolved in the midgut of the insect and viruses are absorbed by the epithelial cells of the gut (α phase) (Summers, 1977; Luckow, 1991; O'Reilly et al., 1992; Volkman, 1997; Winstanley and O'Reilly, 1999). The viruses migrate to the nucleus of the epithelial cells. They get uncoated, enter into the nucleus and their genes are transcripted and translated by the host cells.

During the β phase (4 to 8 h post infection), the infected cells undergo changes such as cytoskeleton rearrangement and chromatin condensation. After that (γ phase, 8 to 24 h.p.i.) the virus begins to replicate its DNA and expresses its proteins. Nucleocapsid assembly begins in the nucleus. The nucleocapsides leave the nucleus, bud though the cell membrane into the hemolymph, acquiring a cell-menbrane derived envelope in the process. After being exocytosed, budded viruses are transported through the hemolymph to carry the disease to other larval tissues.

A secondary infection cycle begins by the entrance of a virus into another cell of the insect's body (phase δ, 24 to 96 h.p.i.). Like during the primary infection cycle, the virus is transported to the nucleus, gets uncoated and enters into the nucleus. The virus DNA is replicated and the viral proteins are produced. During this secondary cycle, proteins constituting the crystalline matrix of the OV are produced, and among them, polyhedrin. The nucleocapsids are assembled before being enveloped by the polyhedrin matrix to form OV. Whereas extracellular BV level reaches its maximum by 2 days post infection, OV continue to accumulate into tissues for 4-6 days (Glazer and Nikaido, 1995). During this time, epithelial cells continue to produce BV.

The second cycle ends with the synchronized lysis of the cells infected during that cycle (between 96 to 120 h.p.i.). The tissues of the larval body are destroyed, the larvae die, and the OV are spread into the environment where they can stay alive for several years before being ingested by other larvae (Volkman, 1997). In vitro, the life cycle of wild-type viruses is similar except that cells must be infected directly by non-occluded viruses through the addition of BV virus or of previously alkaline digested OV. In vitro infected insect cells are able to produce both BV and OV (Glazer and Nikaido, 1995).

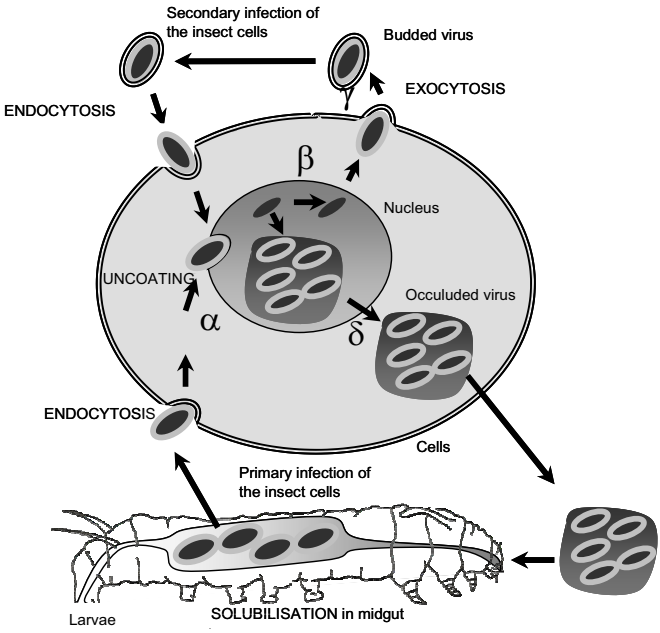


Figure 1-3: Life cycle of wild-type MNPV baculovirus in nature

1.4.3. RECOMBINANT BACULOVIRUSES

In nature, polyhedrin is necessary to form OV that are the only phenotype able to stay alive in the environment (Glazer and Nikaido, 1995). In vitro, the polyhedrin is not necessary for virus survival because BV can readily infect invertebrate cells and replicate into them in vitro. Polyhedrin is a protein of 29 kDa, i.e. the major protein of the OV that could represent 30-50 % of the total larvae protein mass at the end of the infection (O'Reilly et al., 1992). In vitro, up to 1 mg of protein could be synthesized by 1-2x10^6 infected cells (Crossen and Gruenwald, 1997). Recombinant baculoviruses are constructed by the replacement of the polyhedrin gene (*polh*) by a foreign gene controlled by the very strong late *polh* promoter, which thus allows the production of r-protein at very high yield (Smith et al., 1983; Merrington et al., 1997).

The infection phase of insect cells in vitro by a recombinant baculovirus is quite similar to that by wild-type virus (Figure 1-4). Cultures are directly infected by BV and virus progeny are not occluded because the *polh* gene has been removed. During the early phase (0 to 6 h.p.i.), the recombinant virus enters by endocytosis, the nucleocapsid get uncoated and enters into the nucleus (O'Reilly et al., 1992). Infected cells undergo cytoskeletal modification and chromatin condensation. During the late phase (6 to 20-24 h.p.i.), extensive DNA replication and protein production occurs to produce more

BV. Nucleocapsids are budded from the cytoplamic membrane. The very late phase of the infection (20-24 to 96-120 h.p.i.) is characterized by the late gene expression necessary for the production of OV. When a foreign gene has replaced the polyhedron-encoding gene under the control of the *polh* promoter, the production of BV is greatly reduced and cells produce high levels of foreign gene product instead of polyhedrin (O'Reilly et al., 1992).

The *polh* gene product or its replacement starts being expressed from 18 h.p.i. but is overexpressed from 48 to 120 h.p.i. (Palomares et al., 2006) The foreign gene accumulates into the cells during the final portion of the late phase. At the end of the infection, when the cytopathic affects are so extensive that cell lysis occurs, the r-protein is harvested from the culture medium together with virus and cell fragments. When the number of virus divided by numbers of cells (multiplicity of infection: MOI) is less than one, two or more infection cycles are required to infect all cells in the culture.

Gene coding for other viral proteins have also been replaced instead of the polyhedrin gene, for the construction of recombinant baculovirus vectors. The p10 is a protein produced during the late infection phase and is associated with a fibrous structure necessary for the precondensation of OV (O'Reilly et al., 1992). This fibrous network composed of p10 proteins contributes cell lysis (Williams et al., 1989). The use of the *p10* promoter to express a foreign gene helps to delay cell lysis and thus increase the time of production. Moreover, the use of the *p10* promoter instead of the *polh* leads to improved glycosylation, but the overall r-protein yields are usually lower than those obtained with the *polh* promoter (DiFalco et al., 1997; Merrington et al., 1997).

The use of early promoters such as *da26, egt, etl, lef-7, ie-0, ie-1, ie-2, me53, pe38, p35, p39* (major nucleocapsid protein), *p6.9* (core capside protein), ect. allows a more rapid production of r-protein and its harvest from the cells without waiting for cell lysis (Palomares et al., 2006). The use of these earlier promoters leads, however, to lower production yields but allows more extensive and better post-translational modifications (Jarvis et al., 2001). The *bbp* (basic protein promoter) production can start as early as 6 h.p.i. (Lawrie et al., 1995) and, at least in one case, the use of this promoter resulted in the highest product yields reported, i.e. higher than both with *p10* and *polh* promoters (Bonning et al., 1994). Today, the majority of BEVS are constructed based on the strength and timing of the *polh* or *p10* late gene promoters.

The use of baculovirus expressing multimeric proteins behind the same promoter or two different promoters (e.g. *p10* and *polh*) allows the expression of several proteins during the same production with high yield. The use of a coding region for a homing sequence placed between the promoter and the foreign gene allows the expression of a secreted r-protein (Davis et al., 1992; Sakaguchi, 1997).

Construction of a r-baculovirus vector involves four steps: construction of a transfer vector, recombination between vector and baculovirus, selection of r-baculovirus among the non-transfected parental virus and amplification of r-virus (Merrington et al., 1997). The gene of interest is inserted by conventional methods into a plasmid as transfer vector under the control of a promoter and flanked by sequences homologous to parental DNA in a locus not essential for virus replication. Secondly, the parental DNA is co-transfected with the transfer vector into insect cells (generally Sf-9 cells) by any of the various classical methods. Recombination occurs between homologous sequences of the transfer vector and the parental virus resulting in the production of r-baculovirus. Subsequently, r-baculovirus are selected from original parental virus (Luckow, 1991; O'Reilly et al., 1992).

Originally, selection of recombinant virus with replacement of the *polh* gene used to be undertaken by visual inspection of virus plaques for absence of polyhedra. This laborious method was soon abandoned and methods of selection using a transfer vector containing the lacZ gene were used. After recombination, in the presence of the chromogenic substrate (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) X-gal, the r-virus plaques appear coloured in blue, while parental virus plaques are colorless (O'Reilly et al., 1992). However, these selections remain difficult to apply.

Therefore, to reduce these selection steps, linearized viruses have been introduced. A restriction enzyme site was added on the parental viruses at the insertion locus. These vectors are almost non-infectious. But when cells are co-transfected with a transfer vector based on the same locus, recombination generates a recirculisation of the virus that endows it with a high infectivity (Kitts, 1996). The efficiency of this strategy was improved by the construction of linearized viruses with defective essential genes. These linear parental viruses are completely non-infectious (Merrington et al., 1997). Infectivity is recovered upon recombination of this virus with a transfer vector containing the intact essential gene (or its previously portion) in addition the foreign gene. Today, there is a high variety of viruses constructed with this method, displaying recombination frequencies of almost 100%. The final step after selection is the amplification of the selected r-baculovirus. Successive amplifications step are usually carry out in Sf-9 cells with a MOI of 0.1 until viral stocks reach 1.10^8 pfu (plaque forming unit)/ml. Stocks are titrated and

maintained for long term storage at 4°C, -80°C or in liquid nitrogen depending on the frequency of their intended use (O'Reilly et al., 1992).

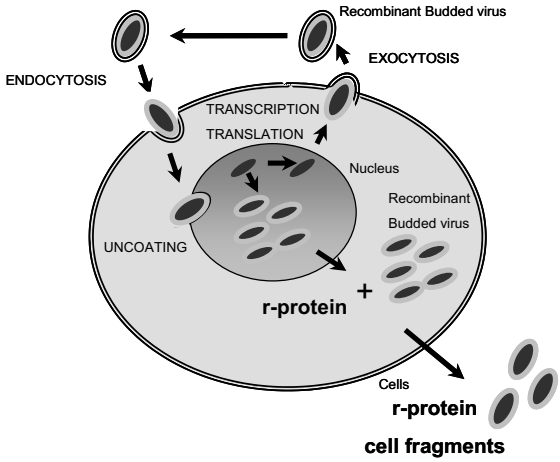


Figure 1-4: Infection cycle of in vitro recombinant baculovirus.

1.4.4. The Baculovirus expression vector system as a technology platform in biotechnology

Compared to other systems, the IC-BEVS has several advantages (Table 1-3). Recombinant baculoviruses are genetically stable due to their small, double stranded circular DNA (Maeda et al., 1985; Smith et al., 1985). Their stocks can be amplified several times (Ross et al., 1998). They are able to express foreign genes of prokaryotic, eukaryotic or viral origin (O'Reilly et al., 1992). Yield of production of functional heterologous r-protein is typically higher than for mammalian cells. Although industrially attained product titers are not often in the public domain, the highest reported product titer using IC-BEVS in industrial application is 300 mg/L (human collagenase) with High-Five cells and 80 mg/L (human propolipoprotein AI) with Sf cells, which is similar to the highest reported level obtained with mammalian (CHO) cells (Schmidt, 2004). Baculovirus vectors allow the expression of large foreign gene products from 17 to 110 kDa (Maiorella et al., 1988).

Furthermore, this system is adapted for simultaneous expression, up to five foreign gene, using a single recombinant baculovirus (Zavodszky and Cseh, 1996). The recombinant viruses produced using these transfer vectors have facilitated the study of complex protein assembly, by co-expressing protein subunits in the same host cells. Co-infection with several recombinant viruses is also possible to express different genes (Rhodes, 1996; Kost et al., 2005). Insect cells are well adapted to serum-free suspension

cultivation and can reach higher cells densities compared to mammalian cells (Kost and Condreay, 1999).

They are well adapted to bioreactor cultivation and scale-up (up to 1000 L have been reported) (Agathos, 1994). Insect cells are able to bring about eukaryotic post-translational modification of heterologous proteins (N- and O-glycosylation, phosphorylation, fatty acid acylation, sialylation, α-amidation, N-terminal, acetylation, carboxymethylation, isoprenylation) (Betenbaugh et al., 1996; Joshi et al., 2000; Hollister et al., 2002; Palomares and Ramirez, 2002; Joosten and Shuler, 2003; Palomares et al., 2003)

Moreover, baculovirus-infected insect cells are able to effect folding of heterologous proteins, and peptide cleavage, although these r-proteins exhibit differences from their authentic counterparts. High-Five cells are supposed to carry out better post-translational modifications than Sf-9 cells with a higher percentage of complex glycoforms and even some sialylation (Joshi et al., 2000; Joosten and Shuler, 2003).

Nowadays, the construction of a r-baculovirus is simple and rapid (Philipps et al., 2005). Transfection, selection and amplification of viral stock steps are not too laborious. Moreover, this transient expression system produces r-proteins in a short time. Baculoviruses are non-pathogenic for human but only for insect cells, while the latter do not carry human oncogenes and hence, they do not present a cancer risk to humans (Stacey and Posee, 1996; Galbraith, 2002). The BEVS allows the production of proteins or biopesticides in vitro in cultured insect cells or in vivo in larvae. Although the production yield from larvae is high, r-protein produced from larvae are not common because following the lysis of the caterpillars, there is a need for several purification steps to obtain a pure protein. Moreover, in vivo production requires high expertise in growing and maintenance of larvae, an art that is not as amenable to automation, as in vitro insect cell culture.

However, the BEVS has also some drawbacks (Table 1-3). Firstly, the BEVS is a transient lytic system. Cell lysis occurring at the end of the cultivation could compromise the quantity and quality of the r-protein produced (by release of proteases or other factors). Moreover, the lytic nature of this system complicates the purification steps following the harvest. The infection process produces virus fragments associated with the r-protein that also complicate the purification steps (O'Reilly et al., 1992). Secondly, post-translational modifications by insect cells are different from those by mammalian cells (Palomares and Ramirez, 2002; Jarvis, 2003; Joosten and Shuler, 2003). Although these generally affect the micro-heterogeneity of the glycoforms, they may alter protein activity.

Table 1-3: Main expression system of recombinant proteins

Host		Time for expression	Cost of technology	Post-translational modification	Cell density	Growth rate	Protein folding	Secretion of protein	Protein expression level	Other Benefits	Drawbacks
Cultivation of procaryotes	**Bacteria**	Short	Low	No	Very high	Very high	Poor	Poor	0.5 - > 5 g/l	• Well known genetics • Multitude of vectors	• Formation of toxic waste products,incl. endotoxin in culture (enterobacteria)
Cultivation of unicellular eukaryotes	**Yeast**	Short	Low	Simple	High	High	Moderate	Moderate	0.2 - > 2 g/l	• Well known genetics • No toxicity	• Oligomannosidic glycosylation
In-vitro cultivation of cells of higher eukaryotes	**Mammalian cells**	Long	Expensive	Complex, close to human	Low	Low-Moderate	Very good	Very good	2- > 500 mg/l	• Glycosylation nearly human • Production of big proteins • Stable expression	• Low gene expression level • Expensive media • Fragile cells
	Insect cells	Rapid	Moderate	Complex	Moderate	High	Good	Good	0.2 - > 1 g/l	• Production of big proteins • Simultaneous expression of several proteins	• Fragile cells • Transitory (lytic) expression system (BEVS) • Simultaneous production of baculovirus DNA and proteins • Incomplete glycosylation
Transgenic eukaryotes	**Transgenic animal**	Very long	Expensive	Complex, close to human	N/A	N/A	Very good	Very good	0.5- > 2 g/l	• Continuous production of secreted protein into milk or eggs	• Initial investment very expensive • Risk of contamination

Due its transient nature, the duration of protein secretion with the IC-BEVS is shorter than with mammalian cells that express constitutively secreted r-protein (Schmidt, 2004). Finally, as stated previously, mutant viruses tend to appear in viral stocks passaged over many times. These so-called 'defective infecting particles' (DIP) are incapable of replicating by themselves but may replicate and alter the outcome of the infection when they co-infect cells together with a non-defective r-baculovirus (Wickham et al., 1991). Baculoviruses cultivated typically at 27°C may not be appropriate for the expression of cold-sensitive proteins derived from organisms with a optimal temperature of > 27°C (O'Reilly et al., 1992). Finally, as seen before, baculoviruses are generally engineered with a late promoter that becomes active only at the end of the infection, hence the full expression potential of the vector may not be exploited.

In conclusion, the IC-BEVS produces higher titers of a given r-protein than mammalian cells but with differences in post-translational modifications compared to mammalian host cell systems. Compared to bacteria and yeast, proteins produced using the IC-BEVS provides more complete eukaryotic post-translational modifications, but it reaches lower production levels produce less. Thus, the IC-BEVS technology constitutes a platform that is attractive for applications when the benefits are higher than drawbacks.

1.5. INSECT CELL CULTIVATION

1.5.1. INSECT CULTURE CONDITIONS

Despite the general similarities with mammalian cell cultivation, the cultivation of insect cells has some notable differences (Table 1-4). Most insect cell lines are adapted to growth in suspension, but non-adherent Sf-9 and High-Five insect cells can be also immobilized with good results (Wu and Goosen, 1996; Ikonomou et al., 1999).

1.5.1.1. Effect of temperature on insect cells

Insect cell lines can be cultivated over a temperature range of 25-30°C (Agathos et al., 1990). Nevertheless, the optimal temperature in terms of specific growth rate and final cell density is 27°C for the Sf-9 cells (Reuveny et al., 1993b) and the High-Five cells (unpublished result). However, Gerbal et al. (2000) have adapted Sf-9 cells to culture at 37°C over long-term passaging, thus showing that insect cells can become thermo-tolerant. At 25°C, the specific growth rate (μ) was found to be reduced while at 30°C, the cells viability and the maximal cell density decreased (Reuveny et al., 1993a).

Table 1-4: Comparison of cultivation in vitro of insect and mammalian cells.

	Mammalian cells	Insect cells
Culture conditions		
Temperature of growth	35-37°C	22-29°C
Temperature of protein production	33-37°C	25-29°C
Optimum pH	7.0-7.3	6.2-6.3
Media osmolality	280-330 mOsm/kg	320-375 mOsm/kg
Growth atmosphere	5 % CO_2	0-5 % CO_2
Media requirements		
Glucose	Moderate (10-20 mM)	High (10-50 mM)
Amino acids	Moderate (1-2 mM)	High (2-10 mM)
Lipids	Often	Yes
Buffer	CO_2/bicarbonate or HEPES or MOPS	Phosphate
Surfactants	Yes (for suspension)	Yes (for suspension)
Serum	Not necessary	Not necessary
Undefined extracts	Less often	More often
Sensitive to thermal shock	Weak	Moderate
Sensitive to change of pH	High	Weak
Sensitive to osmotic shock	Weak	High
Sensitive to change of DO	High	Moderate
Sensitive to ammonia accumulation	High	Moderate
Sensitive to lactate accumulation	Weak	High
Adherent cell cultivation		
Attachment to cell surface	High	Weak
Detachment from cell surface	Trypsinization	Gentle agitation
Contact inhibition	High	Weak
Versatility of suspension/attachment	No	Yes
Suspension cultivation		
Growth rate	~0.017-0.025 h^{-1}	~0.018-0.028 h^{-1}
Dependence of growth on inoculum's size	Yes	Yes
Cell size upon suspension cultivation	10-12 μm	12-15 μm 14-18 μm when infected

The increase of the temperature of cultivation from 22 to 30°C is known to increase the growth rate and the consumption of oxygen and glucose by Sf-9 cells, their cultivation at 35°C decreases these parameters (Reuveny et al., 1993a). During infection, the number of occlusion bodies produced by Tn-368 cells was found to increase at 25 and 32°C compared with 28°C (Hink and Strauss, 1976). Increasing the temperature from 22 to 27°C was also reported to increase the production of β-gal by Sf-9 cells and to increase the proportion of the product released outside the cells, but at temperatures higher than 27°C, productivity decreased (Reuveny et al., 1993a). A temperature increase from 25 to 31°C resulted in a drop in viability of Bm5 cells and an earlier production of r-protein (Andersen et al., 1996).

The temperature was also reported to influence the glycosylation potential in IC-BEVS, lowering the temperature of infection from 28 °C to 24 °C or even 20 °C, resulted in more complete glycosylation of r-proteins in the High-Five cell line (Donaldson et al., 1999).

1.5.1.2. Effect of pH on insect cells

Concerning pH, values between 6.0 to 6.8 are required for the growth of various insect cell lines (Sohi, 1980). The use of phosphate buffer is therefore fully adequate and there is no need to employ CO_2/bicarbonate buffer system commonly used in mammalian cell culture.

The optimal pH was found to be between 6.1 and 6.3 for Bm-5 cells (Zhang et al., 1994); from pH 6.2 to 6.3 for High-Five cells (Drugmand et al.,2005, This thesis, Chapter 3); from pH 6.2 to 6.4 for Sf-9 cells (Hensler et al., 1994) and from pH 6.0 to 6.25 for Tni-368 cells (Hink and Strauss, 1976). At lower or higher pH, a lengthening of the lag phase and a decrease of the growth rate and maximal cell density were observed. However, there was no difference in the numbers of apoptotic cells of Sf-9 or High-Five cultivated at 6.0 or 6.5 (This thesis, Chapter 3). A pH value of 6.2 is generally used for Sf-9 and High-Five cells growth in controlled bioreactors. The same pH is generally used both for the growth and the infection phases. However, the influence of medium pH on r-protein production has not been investigated in detail until now.

Insect cell lines are less sensitive to variation and increase of osmolality than mammalian cells (Yang et al., 1996). They are able to stay alive in a medium whose osmolality varies from 250-450 mOsm/kg. However, the typical medium osmolality for Bm, Sf and Tni cell lines adapted to suspension cultivation is between 320-385 mOsm/kg (Agathos et al., 1990; Zhang et al., 1994), compared to 280-320 mOsm/kg for mammalian cell lines (CHO, BHK, hybridoma).

1.5.1.3. Effect of dissolved carbon dioxide on insect cells

As noted before, in contrast to mammalian media that require a CO_2/bicarbonate buffer, insect cell media are usually buffered with phosphate buffer and do not require a CO_2 incubator. However, caution must be applied when selecting an incubator for insect cell cultivation because the lowest temperature maintained by many incubators is 5°C above ambient temperature (O'Reilly et al., 1992).

In industry very small bubbles of air or, even, pure oxygen are often used for large-scale mammalian and insect cultures, in order to improve the efficiency of oxygen transfer (Marks, 2003). Such small bubbles lead to almost complete dissolution of oxygen that, in turn, is transformed metabolically to CO_2 (1 mole CO_2 produced per mole O_2 consumed, Kamen et al., 1991). Unfortunately, they cannot prevent the accumulation of CO_2 in the bioreactor, especially in high-density cell cultures (Mostafa and Gu, 2003). Mitchell-Logean and Murhammer (1997) showed that CO_2 can accumulate in Sf-9 and Tn-5 cell cultures in a lab-scale bioreactor, to growth inhibitory levels (~24 mM) unless the bioreactor headspace was purged. Garnier et al. (1996) have shown that such a CO_2 accumulation caused a fall of the level of r-protein produced by baculovirus infected Sf-9 cells by almost 70 % in a 110 L reactor compared to 70-ml spinner flasks. We have recently shown that High-Five cells cultivated in a fixed-bed reactor are highly sensitive to CO_2 accumulation and recommended to avoid a partial pressure of CO_2 higher than 20 % of gas saturation (This thesis, Chapter 6).

It is currently thought that at least one mechanism of cell toxicity due to CO_2 accumulation may be due to the stress from the formation of carbonate anion CO_3^{2-} (Goss et al., 1999) or of peroxymonocarbonate anion HCO_4^- (Richardson et al., 2003). In addition CO_2 accumulation results in a decrease of pH or in increased medium osmolarity (due to addition of base to control the pH): upon dissociation of $CO_2 + H_2O$ into H^+ and HCO_3^-, the corresponding HCO_3^- and the alkali (e.g. KOH or NaOH) added to control medium pH leads to increased osmolarity. However given the relative resistance of insect cells to osmotic pressure, the adverse effect of CO_2 may be due more to oxidative stress and less to osmotic stress.

1.5.1.4. Effect of dissolved oxygen on insect cells

Insect cells are strict aerobes with a high TCA activity. They have a higher specific consumption rate of oxygen than mammalian cells (which have a higher specific rate of production of lactate). Although insect cells need more oxygen than mammalian cells, they can be cultivated in the same bioreactor types with similar gas process control. Such control schemes (as PID: Proportional-Integral-Derivative control) ensure not only the sufficiency of DO levels for growth and product formation (avoidance of oxygen limitation) but also that there is no toxicity associated with excessive oxygenation, witch typically produces harmful reactive oxygen species (ROS). Nevertheless, the parameters of oxygen transfer rate (OTR) monitoring must be adapted to insect cell cultures. Monitoring of specific

oxygen uptake rate (OUR) in insect cell bioreactors provides on-line information on cell density during the growth phase and could provide a quantitative indication of cellular activity in baculovirus infected cells (Wong et al., 1994).

Various studies have investigated the effect of DO levels on cell growth but considerable discrepancies exist among published reports regarding optimal DO ranges for growth and production. In early studies, Hink and Strauss (1980) and Hink (1982) found that the specific growth rate (μ) of Tn-368 cells was similar at all DO levels (15 – 100 % of air saturation). However, in 1991, Jain et al. demonstrated that DO levels had a significant effect on the μ of Sf-9 cells: at DO values equal to 10 and 110 % of air saturation, the μ was ca. 25 % lower than at a DO of 65 %. Gotoh et al. (2004) demonstrated that the specific rate of consumption of oxygen (q_{o2}) by Sf-9 cells varied with temperature and DO and was described by a Monod-type equation.

During the growth phase, insect cells are not very sensitive to variations in the concentration of dissolved oxygen (DO) and can grow optimally in a wide DO range from 30 to 100% (Palomares and Ramirez, 1996; Schmid, 1996). DO values in insect cell cultivation have generally been between 10 to 30 % of air saturation for airlift cultivation whereas 40-60 % are the levels usually employed in stirred tank reactors (STR) (Schmid, 1996). In growth phase, High-Five cells have a slightly higher specific consumption rate of oxygen than Sf-9 cells (Table 1-2) but Sf-9 cells appear to be better able than High-Five cells to protect themselves from oxidative damages (Saarinen and Murhammer, 2002).

On the contrary, during the infection phase, insect cells are more sensitive to DO (Cruz et al., 1998). In fact, infected Bm5 (Zhang et al., 1994), Sf-21 (Deutschmann and Jäger, 1994a) and Tn-368 cells (Schmid, 1996) are more sensitive to DO than Sf-9 (Hensler and Agathos, 1994; Agathos, 1996) and High-Five cell lines (Donaldson and Shuler, 1998b). Moreover, during this phase, insect cells have a higher OUR than uninfected cells (Table 1-2) (Hensler and Agathos, 1994; Kamen et al., 1996). Infected High-Five cells consume oxygen at a specific rate 2 to 5 times that of virally infected Sf-9 cells (Rhiel et al., 1997; This thesis, Chapter 4). Immediately after infection, for both cell lines, a rise of this OUR could be observed (Wong et al., 1994; Palomares and Ramirez, 1996). Palomares et al. (2004) demonstrated that the detection of this maximal OUR in Sf-9 cells could be used to estimate the best time of harvest of r-protein (between 24 and 36 h after maximal OUR).

Infected cells tend to require low DO to sustain high productivity (Taticek and Shuler, 1997; Cruz et al., 1998). Blanchard and Ferguson (1992) found the highest titer of r-protein obtained with baculovirus infected Sf-9 cells at 50 % DO. Reuveny et al. (1993) found that Sf-9 produced more β-galactosidase (β-gal) under conditions were DO was

supposed to be not limiting. Contrary to the above studies, Hensler and Agathos (1994) observed no effect on the β-gal produced by Sf-9 cells over a wide range of DO levels (5 to 100 %). In 1996, Schmid summarized previous knowledge on the effect of DO on insect cells and found that Sf-9 cells have the highest expression of IFN–γ (ca. 10 μg/ml at 120 h.p.i.) at a DO between, 10 and 30 %. In all investigations of the effect of DO levels on r-protein production significant differences in product yield have been observed, except in the work of Hensler and Agathos (1994). However, this is not surprising, given the different media, cell lines and methodologies employed in the different reports.

Concerning r-protein quality, Zhang et al. (2002) have demonstrated that infected Sf-9 and High-Five cells produce relatively more highly glycosylated secreted alkaline phosphatase (seAP) at a DO equal to 50% of air saturation than at either 10 % or 190 % of air saturation. In summary, the effect of DO on quality and quantity of r-protein produced depends on the cell line, on the virus and on the r-protein (Schmid, 1996; Taticek and Shuler, 1997), but there is still a lack of complete understanding of the effect of this process parameter.

1.5.1.5. Effect of shear stress on insect cells

Insect cells cultivated in bioreactor are subjected to relatively high shear stresses. The cell damages are a function of the type, duration and magnitude of the hydrodynamic forces caused by agitation and sparging. Insect cells that usually required high agitation speed in shake-flasks to sustain oxygenation (100-150 rpm) were found to be less sensitive to shear stress than mammalian cells (Tramper et al., 1986; Chalmers, 1996). Insect cell have been found vulnerable to laminar shear above 0.59 N/m^2 and to energy dissipation rates above $2.25x10^4$ W/m^3, which is higher than value generated in a classic stirred tank (2 to $3.5x10^3 W/m^3$) or bubble column (0.2 to $2x10^3 W/m^3$) (Palomares et al., 2006). Cell exposure to high levels of hydrodynamic stress are damaged irreversibly and die principally by necrosis (This thesis, Chapter 3). On contrary, a moderate level of stress implies physiological effects that could trigger apoptosis of cells (Tramper et al., 1986; Chisti, 2000; This thesis, Chapter 3).

1.5.2. Insect culture media

Insect cell media are different from mammalian cell media. They contain the same basal elements (carbohydrates, amino acids, salt) but with concentrations adapted to insect cell metabolism. Contrary to mammalian media, they are supplemented with specific additives such as a lipid mixture, to supply to the cells some lipids that they are unable to

produce (Goodwin, 1991). Moreover, surfactants such as Pluronic F-68 are added at ca. 0.1-0.2% (w/v) to protect cells from shear stress occurring in an agitated reactor, and antifoam is also commonly added in sparged reactor cultivation (Murhammer and Goochee, 1990). Phenol Red as pH indicator is absent from insect media, hence they appear usually clear or yellow (due to the presence of serum or peptone hydrolysates). The addition of an antibiotic-antifungal solution that is typically used during the establishment of cell lines, must be avoided during cultivation for safety and purification reasons (O'Reilly et al., 1992).

First insect cells media were developed in the 60's and 70's based on the insect cell hemolymph composition. These basal media such as Grace's medium (Grace, 1962), TNM-FH (Hink, 1970) or TC-100 (Gardiner and Stockdale, 1975) are composed of carbohydrates, amino acids, organic acids, salts and basal mixtures of vitamins supplemented with 5% or 10% foetal bovine serum (FBS). Some media were originally supplemented with hemolymph but serum became the preferred additive.

Serum provides growth factors, proteins with detoxifying and antioxidant effects, carrier proteins and protease inhibitors and protects cells from shear stress. Unfortunately, it is expensive, has lot-to-lot variability, its supply is limited or unreliable and it may contain adventitious agents or contaminants. Moreover, it can interfere with the purification of r-proteins (Ikonomou et al., 2003). Unfortunately, insect cells are unable to grow in these basal media (Grace, TNM-FH, TC-100) if serum is removed: they can stay alive, but do not grow. Thus, these considerations, amongst many others, have motivated the development of serum-free media (Agathos, in press).

Although serum-free medium development can be tedious and costly, in the last several years, substitutes of serum have been sought. Protein hydrolysates (also named peptones) are known to be promising supplements for the large-scale, serum-free culture of animal cells. They are complex mixtures of oligopeptides, polypeptides, vitamins and amino acids produced by enzymatic or chemical digestion of casein, albumin, plant or animal tissues or yeast cells. Hydrolysates have similar protective effects as serum (antioxidant, shear stress, etc.), can supply growth factors and protease inhibitors, can have an antiapoptotic influence and may also have a nutritional role if basal media with lower amino acid content (or no amino acids) are used. Moreover, integration of an ultrafiltration step in media preparation can eliminate hydrolysate lot-to-lot variability and ensure reproducibly good growth of insect cell lines (Ikonomou et al., 2003).

Efforts to eliminate serum from insect cell culture began in the early 80's. Wilkie et al. (1980) developed CDM that was the first serum-free insect cell medium containing

Yeastolate hydrolysate for growth and infection of Sf cell lines. Yeastolate is a yeast extract ultrafiltrated from autolysed yeast biomass that can perform many serum functions. It contains vitamins of the B complex, that are important for the maintenance of insect cell lines (Mitsuhashi, 1989). It is known to extend the growth phase of insect cells (Bédard et al., 1994; Vaughn and Fan, 1997). Moreover, it was identified as essential for the production of some autocrine growth promoting factors by Sf-9 cells (Calles et al., 2006b).

In 1981, Weiss et al. developed IPL-41, a serum-free basal medium adapted to large scale culture. Supplemented with 4 g/l Yeastolate and a lipid mixture emulsified in Pluronic F-68, it supported the culture of Sf-9 cells. In the next few years, modifications of the IPL-41 and CDM supplemented with Yeastolate (Maiorella et al., 1988), egg yolk (Röder, 1982), lactalbumine (Schlaeger, 1996b) or Ex-Cyte (Hink, 1991) were developed to sustains the growth of Sf-9, Sf-21, Tn-386 and SL-2 cells (*Drosophila* Schneider S2 cell line). In 1993, Schlaeger et al. developed the first serum-free medium (SF-1) sustaining high growth rates of High-Five cells, whereas in 1995, Öhman et al. developed a serum-free media (KDM-10) specifically adapted to Sf-9 cells. This SF-1 media contain Primatone. It is an enzymatic digest of animal tissue used as cost-effective supplement which, under serum-free conditions, allows to prolong the stationary phase by delaying the apoptosis of both Sf-9 and High-Five insect cells (Schlaeger, 1996a). Unfortunately, Primatone is of animal origin which may make it less desirable for pharmaceutical applications.

This safety concern has stimulated interest in developing animal-free media. ISYL was the first serum- and animal-free insect media developed for the large scale culture of insect cell (Donaldson and Shuler, 1998a). It contains 4g/l of soy peptone and is based on the IPL-41. It allows to reach high cell densities and r-protein production with High-Five cells. In 2001, Ikonomou et al. developed a serum-free media (YPR media) based on IPL-41 and containing lipids, Primatone and Yeastolate. YPR media is specifically adapted to High-Five cells and allows perfusion cultures with high cell densities and productivities.

A number of commercial media have been specifically developed for Sf and Tni cells and have appeared on the market in the last decade. The media Sf-900 II and Express-Five™ SFM from Invitrogen Gibco (Carlsbad, CA), Insect-XPRESS from Cambrex (Walkersville, MD), EX-Cell 400, 405 and 420 from JRH Biosciences (Lenexa, KS) and HyQ SFX-Insect and HYQ CCM3 from Hyclone (Logan, UT) are now available and used routinely for the cultivation of insect cells (Table 1-5). In addition, several media are specifically developed for transfection (Table 1-5). Most contemporary, insect cell growth media are serum-free and contain yeast, meat or soy hydrolysates, some of them are protein or animal-free. These media, although able to support high cell densities

and protein titers, are expensive, cell-line specific and of proprietary composition. Altought culture conditions are not the same for all lepidopteran cell lines, substantial similarities exist (Agathos, 1996). Insect cell media are usually developed for a unique cell line, or a narrow spectrum of cell lines (e.g. Sf cell lines). Even if lepidopteran cells can stay alive in a non-specific medium, they usually need a specific medium well adapted to their physiology and metabolism to sustain cultivation leading to high densities and to high production levels. Today almost all serum-free media in the public domain are developed for Sf-9 cells, while only the SF-1 and YPR media have been specifically developed for High-Five (Schlaeger, 1996b; Ikonomou et al., 2001).

Table 1-5: Common insect cell media

MEDIA IN THE PUBLIC DOMAIN			
Medium	Features	Cell lines	References
Grace's medium	Needs to be supplemented with 5-10 % (FBS) serum	Invertebrate cell lines	Grace (1962)
TNM-FH	Needs to be supplemented with 5-10 % (FBS) serum	*Trichoplusia ni* cell lines	Hink (1970)
TC-100	Needs to be supplemented with 5-10 % (FBS) serum	Insect cell lines	Gardiner and Stockdale (1975)
CDM	Chemically defined medium Need Yeastolate to allow cell growth	*Spodoptera frugiperda* cell lines	Wilkie et al. (1980)
IPL-41	Chemically defined medium without serum, Need Yeastolate to allow cell growth	Insect cell lines	Weiss et al. (1981)
M-CSF	IPL-41 supplemented with Yeastolate and Pluronic F-68 Developed for large scale	Insect cell lines	Maiorella et al. (1988)
SFM-LP	TNM-FH supplemented with Ex-Cyte VLE and Pluronic F-68	Sf-9 and TN-386 cells	Hink (1991)
EMIGG	Modified CDM supplemented with Yeastolate and Pluronic F-68	Sf-9 cells	Ferrance et al. (1993)
SF-1 (also named IP301)	Chemically defined medium supplemented with Primatone, Yeastolate, lactalbumine, glucose and Pluronic F-68 Developed for large scale	Sf-9, High-Five, SL-2 and SL-3 cells	Schlaeger et al., 1993
KBM-10	Serum-free Developed for metabolic studies	Sf-9 cells	Öhman et al. (1995)
ISYL	modified IPL-41 supplemented with soy hydrolysate	*Trichoplusia ni* and *Spodoptera frugiperda* cells lines	Donaldson and Shuler (1998)
YPR	modified IPL-41 supplemented with Primatone, Yeastolate, Pluronic, lipids, glucose and Gln Developed for large scale	High-Five and Sf-9 cells	Ikonomou et al.(2001)

COMMERCIAL MEDIA			
Medium	Features	Cell lines	Company
Growth media			
Drosophila SFM	Serum-free Proprietary composition	*Drosaphila* cell lines	Invitrogen GIBCO (Carlsbad, CA)
Ex-cell 400	Serum-free, protein-free medium. Hydrolysate source yeast	*Spodoptera* cell lines	JRH Biosciences (Lenexa, KS)
Ex-cell 405	Serum-free Proprietary composition Developed for large scale	High-Five and Sf-9 cells	JRH Biosciences
Ex-cell 420	Serum-free Proprietary composition Developed for large scale	Sf-9, Sf-21 and SL-2 cells	JRH Biosciences
Express-Five	Serum-free Proprietary composition Developed for large scale	High-Five cells	Invitrogen Gibco
HyQ CCM3	Serum-free Proprietary composition	Sf-9 cells	Hyclone (Logan, UT)
HyQ SFX	Serum-free Proprietary composition Developed for large scale	High-Five, Sf-9, Sf-21 and D.Mel(2) cells	Hyclone
Insectagro FIVE	Serum-free and protein-free medium	High-Five	Mediatech Inc. (Herndon, VA)
Insectagro Sf-9	Serum-free and protein-free medium	Sf-9	Mediatech Inc.
Insect Express™ High Five	Serum-free and protein-free medium Proprietary composition	High-Five	PAA Laboratories, GmbH (Pasching, Austria)
Insect Express™ Sf9-S2	Serum-free and protein-free medium Proprietary composition	High-Five	PAA Laboratories, GmbH
Insect-Xpress	Protein-free Proprietary composition Developed for large scale	Sf-9 and Sf-21 cells	Cambrex (Walkersville, MD)
IS-BAC	Serum-free, protein-free medium Proprietary composition	Sf-9, Sf-21 and High-Five cells	Irvine Scientific (Santa Ana, CA)
SF900	Serum-free Proprietary composition	Sf-9 and Sf-21 cells	Invitrogen GIBCO
SF900-II	Serum-free Proprietary composition Developed for large scale	Sf-9 cells	Invitrogen GIBCO
Serum-free insect medium-1	Serum-free Proprietary composition	Insect cell lines	Sigma-Aldrich (Saint Louis, MO)
Serum-free insect medium-2	Serum-free Low protein content Proprietary composition	Insect cell lines	Sigma-Aldrich
Serum-free medium	Serum-free medium Custom-made media	Insect cell lines	Chemicon International (Temecula, CA,)

Transfection media			
BaculoExpress ICM SF-1, 2, 3 and 4	Serum-free Proprietary composition	Insect cell lines	PromoCell GmbH (Heidelberg, Germany)
Bioinsect-1	Serum-free medium Proprietary composition	Lepidopteran cell lines	Biologicals Industries (Kibbutz Beit Haemek, Israel
TriEx	Proprietary composition	Insect cell lines	Merck Biosciences Novagen (Darmstadt, Germany)
ESF 921	Serum-free, protein-free medium Proprietary composition	Insect cell lines	Expression Systems (Woodland, CA)
ESF AF	Animal-free medium Proprietary composition	Insect cell lines	Expression Systems
ExpressSF+	Proprietary composition	Insect cell lines	Protein Sciences (Meriden, CT)
SF IC culture medium	Serum-free medium Proprietary composition	Insect cell lines	Gentaur (Brussels, Belgium)
SF, Met-free IC medium	Serum-free, Met-free medium Proprietary composition	Insect cell lines	Gentaur
SF IC medium-2	Serum-free, low protein medium Proprietary composition	Insect cell lines	Gentaur
Serum-free Insect Virus Production Medium.	Serum-free medium Proprietary composition	Insect cells lines	MP Biomedicals (Irvine, CA)

Amongst commercial growth media, six are specifically developed for High-Five cells; seven for Sf-9 cells and three for both cell lines (Table 1-5). Media developed for High-Five cells are usually richer than those for Sf-9 cells. Usually, Sf-9 cells are able to grow in media developed for High-Five cells but with a higher production of by-products. On contrary, High-Five cells when cultivated in media developed for Sf-9 cells (e.g. Sf900-II) have a reduced growth rate with a very high production of by-products and a decrease yield of production (Rhiel et al., 1997).

1.6. BIOREACTOR CONFIGURATION

The IC-BEVS technology involves two distinct phases of cell culture: at first the cells are cultivated to reach a desired cell density; subsequently, the cells are infected to produce either r-protein or baculoviruses. Infected cells stop growing while uninfected cells continue to proliferate.

Table 1-6 (next pages): Bioreactor operation strategies for recombinant protein production in the BEVS (Modified from Jäger, 1996 and Ikonomou et al., 2003). AchE: *Drosophila melanogaster* acetyl cholinesterase, α-1,3 GT: α-1,3 galactosyltransferase, β-TP: β-trace protein, CAT: bacterial chloramphenicol acetyltransferase, EBNAl: Epstein–Barr virus (EBV)-encoded nuclear antigen 1, EGFR-ED: epidermal growth factor receptor-extracellular domain, hIL-5: human interleukin-5, hNGF: human nerve growth factor, VP2H: structural protein of infectious bursal disease virus, VP6: bovine rotavirus nucleocapsid protein.

Cell line	Bioreactor operating strategy	Medium	X_{max} (10^6 cells/ml) (growth)	X_{max} (10^6 cells/ml) (infection)	Recombinant protein / virus	Maximal Production	Reference
Medium replacement							
Sf-9	Total replacement immediately after infection	TNM-FH with 10% FBS	~5.0	4.6	VP6	800 µg/ml	Caron et al., 1990
	Total replacement prior to infection	Ex-Cell 400	2-3	1.03	β-gal	530 U/ml	Lindsay and Betenbaugh, 1992
	Total replacement prior to infection plus glucose and Yeastolate supplementation	ICSF-WB	4.5	4	Glucocerebrosidase β-gal	>6 µg/ml 3.282 mg/ml	Reuveny et al., 1993
	Total replacement prior to infection	Ex-Cell 401	N.I.	3	β-gal	8×10^5 U/ml	Hensler and Agathos, 1994
	Total replacement immediately after infection	Sf-900 II	9.0	9.0	EGFR-ED	6.4 mg/L	Tom et al., 1995
	Total replacement prior to infection	Sf-900 II	21.6	7.23	β-gal	14.1×10^5 U/ml	Radford et al., 1997
	Partial medium replacement prior infection	TNM-FH with 10% FBS	8	N.I.	β-gal	320 U/ml	Yamaji et al., 1999
Specific nutrient supplementation							
Sf-9	Additions of glucose, glutamine and Yeastolate	IPL-41	15.8	-	-	-	Reuveny et al., 1994
	Glucose (daily) and glutamine (time of infection) feeding	Ex-Cell 401	4.6	2.9	Epoxide hydrolase	0.11 U/ml	Wang et al,. 1993
	Mixture of amino acids and yeastolate ultrafiltrate at time of infection	Sf-900 II	N.I.	6.6	β-gal	240.7 U/ml	Bédard et al., 1994
Sf-21	Elevated glutamine concentration and oxygen environment	Ex-Cell 400	N.I.	7.0 6.4	β-gal seAP	855 IU/ml 26.2 U/ml	Taticek and Shuler, 1997

N.I.: no information

Fed-batch culture							
Sf-9	Multiple additions of glucose, glutamine, lipids and Yeastolate	Ex-Cell 400	12	5	HNGF	20 mg/L	Nguyen et al., 1993
	Single addition of amino acids, lipids Yeastolate and trace elements	Sf-900 II	16-23	~7	β-gal	~270 U/ml	Bédart et al., 1994
	Single addition of glucose, amino acids, lipids, Yeastolate, vitamins, metals	Sf-900 II	30	11-12	β-gal	895 U/ml	Bédard et al., 1997
	Additions of glucose, glutamine, Yeastolate, lipids and amino acids	IPL-41	~4	4.2	β-gal	235 U/ml	Wu et al., 1998
	Single addition of yeastolate, amino acid mixtureand lipids	Sf-900 II	13-18	14.5	β-gal	$14.7x10^5$ U/ml	Chan et al., 1998
	Continous addition of glucose, amino acids, lipids, Yeastolate, vitamins, metals	Sf-900 II	52	14-17	β-gal	430 U/ml	Elias et al,. 2000
	Additions of amino acids and Yeastolate	Grace's medium with Yeastolate	6	-	-	-	Kim et al., 2000
	Pulse additions of complete medium, glucose, glutamine, Yeastolate, Primatone	YPR	12	15.6	β-gal	120 U/10^6 cells at $11.6x10^6$ cells/ml	This Thesis, Chapter 5
Sf-21	Addition of glucose, Yeastolate and amino acids	TNM-FH with 10% FBS	19	17	hIL-5	3.5×10^6 U/ml	Chiou et al,. 2000
HzAm1	Optimization of feed medium using a genetic algorithm	HyQ-CCM3	19.5	-	–	-	Martejin et al., 2003
High-Five	Additions of glucose, glutamine, Yeastolate, lipids and amino acids	IPL-41	~4	3.8	β-gal	198 U/ml	Wu et al., 1998
	Glucose and glutamine additions based on pH increase	Ex-Cell 401	N.I.	2	VP2H	54 mg/L	Wang and Doong, 2000
	Pulse additions of complete medium, glucose, glutamine, Yeastolate, Primatone	YPR	6.5	6.7	β-gal seAP	687 U/10^6 cells at $4.5x10^6$ cells/ml 25.6 U/10^6 cells at $4.5x10^6$	This Thesis, Chapter 5

N.I.: no information

Cell line	Bioreactor operating strategy	Medium	X_{max} (10^6 cells/ml) (growth)	X_{max} (10^6 cells/ml) (infection)	Recombinant protein / virus	Maximal Production	Reference
Two stage CSTR							
Sf-9	Two stage CSTR	SF900-II	N.I.	1	EBNAI	133 µg/10^6 cells	Meij et al., 2000
Sf-21	Two stage CSTR	TNM-FH with 10 % FBS	9.5-10.5	8.6-11.5	polyhedra	15x10^6/ml	van Lier et al., 1990
Sf-21	Two stage CSTR	TNM-FH with 10 % FBS	N.I.	N.I.	β-gal	6 U/10^6 cells	van Lier et al., 1991
Bm5	Two stage CSTR	IPL-41 with 10 % FBS	3.5	2.7	CAT	255 mg/L	Zhang et al., 1993
Perfusion culture in CSTR							
Sf-9	External hollow fibre	IPL-41	9.5	-	-	-	Reuveny et al., 1992b
	Internal PTFE hollow fibre	TC-100 with 10 % FBS	10	10	AcMNPV	N.I.	Klöppinger et al. 1991
	External tangential filter	TNM-FH	15	12	N.I.	N.I.	Massie et al., 1992
	External hollow fibre	Excell 400	30	11	N.I.	N.I.	Cavegn and Bernard, 1992
	External tangential filter	TNM-FH with ≤1% FBS	15	12-15	VP6	200-250 µg/10^6 cells	Caron et al., 1994
	External tangential filter	ICF-WB	10	-	-	-	Reuveny et al., 1994
Sf-21	Internal polypropylene membrane	modified IPL-41/TBP with 5% FCS	20	-	-	-	Deutschmann and Jäger, 1991
	Internal polypropylene membrane	IPL-41/TBP with FBS and Excell 401	55	20	AcMNPV	N.I.	Jäger et al., 1992
	Internal polypropylene membrane	N.I.	~20	~10	α–1,3 GT	100 U/day	Jäger et al., 1996
	Internal polypropylene membrane	modified IPL-41/TBP with 5% FCS	55	-	-	-	Deutschmann and Jäger, 1994a and b
	External flat menbrane	TC-100 with 10 % FBS	10	8	N.I.	N.I.	Hellenbroich, 1995
SL-2	External polypropylene membrane	M3 + FBS	34	-	-	-	Shütz et al., 1991
SPC-Mb92	External hollow fiber module	Not reported	50	29.6	AchE	43.3 mg/L	Deramoudt et al., 1994
High-Five	Internal polypropylene membrane	modified Ex-Cell 401	N.I.	4	βTP	500-1200 mg/L	Chico and Jäger, 2000
	External hollow fiber	YPR	15.5	-	-	-	Ikonomou et al., 2003

N.I.: no information

Unfortunately, the culture conditions allowing the attainment of high cell densities in the growth phase are quite different from conditions compatible with high production during the infection phase. The pH, temperature and media conditions to sustain high cell densities are well established. During the growth phase, batch culture is the principal mode of culture in which cell growth rate is function of the cell line, the medium and culture conditions (Schlaeger, 1996b). In this phase, physical and chemical shocks must be kept at the minimum.

In addition to these factors, productivity of infected cells is a function of several more factors such as virus construction (Possee, 1997), number of passages of the virus (Krell, 1996), Cell Density at Infection (CDI), Multiplicity Of Infection (MOI) (Chico and Jäger, 2000), "culture age" (Ikonomou et al., 2003), cell state (Wong et al., 1996), state of medium depletion (Jäger, 1996).

The fraction of the population of cells initially infected by the virus is the main factor affecting cell productivity. This fraction of population is a function of the MOI, which corresponds to the number of infectious particles (PFU) divided by the numbers of cells, and follows a Poisson distribution given by the following equation (Equation 1-1). In this, *p* is the probability of a cell being infected by *r* virus and MOI is expressed in PFU/cells (O'Reilly et al., 1992).

$$p(r) = \frac{MOI^{r}.e^{-MOI}}{r!} \qquad \text{(Equation 1-1)}$$

Various papers in the 90's, have studied the combined effect of TOI and MOI on productivity. Infection using high MOI (> 1) implies that infection is synchronized (all cells are infected at the same time), while with MOI < 1, all cells are not simultaneously infected and several cycles of infection are required to infect all the cell population. High MOI allows a short production period with high productivities. Low MOI is used for virus amplification and for applications where viral stocks are low (O'Reilly et al., 1992). Unfortunately, unsynchronised infections increase the time of harvest, the production of defective particle and the exposure of r-protein to proteases (Agathos, 1996).

However, different opinions exist on this point. Some authors consider that a MOI between 1 to 20 leads to essentially similar levels of productivity (Maiorella et al., 1988) while others contend that a MOI close to 1 is better (Agathos, 1996). Although previous attempts to predict the effect of MOI (and TOI) on r-protein production based on simulations (Licari and Bailey, 1992) have been borne out (Wong et al., 1996), the phenomena are complex and MOI optimization is not straightforward. To conclude, MOI

optimization depends on the virus, the cell line, the media/nutritional conditions and the physiological stage of cell at the infection.

The second main factor affecting production is the "cell density effect" (that depends on the CDI). It consists in the reduction of the specific productivity when cell concentration at infection is above 3-4x10^6 Sf-9 cells/ml (Elias et al., 2000) or above 5-6x10^6 High-Five cells/ml (Ikonomou, 2002; This thesis, Chapter 5). This is thought to be due to nutrient depletion in the medium during infection, although its current understanding is far from complete. For instance, Doverskog et al. (1997) have shown that the drop in productivity of Sf-9 cells occurs prior to nutrient depletion. However complete medium replenishment or selected feeding of nutrients seems to counteract the cell density effect (as reviewed by Ikonomou et al., 2003), lending considerable support to nutrient depletion as at least one factor associated with the effect.

Generally at high density and high MOI, infection is more rapid. Cell productivity is also affected by the cells physiological state. In fact, apoptotic cells are less productive than viable cells in the growth phase. Finally, the effect of TOI (also named "culture age"), that is the duration of the culture from inoculation to infection, directly depends on the growth rate of cells, on the inoculation cell density and on the CDI to reach.

Hence, infections that aim to reach high r-protein production must be as synchronized as possible, applied when the cells are highly viable in exponential phase and medium depletion must be compensated with appropriate nutrient feeding.

1.6.1. Medium replacement

Due to the depletion of medium ingredients and the empirically observed cell density effect, infection of simple batch cultures is not optimal for high protein production levels. The first approach to counteract this problem has been the total or partial medium replacement at the time of infection. Medium replacement could be applied before or shortly after the infection and has been found efficient to recover high specific production at high cell density (reviewed by Ikonomou et al., 2003). Moreover, the use of a cell retention system or centrifugation system to separate the cells from the exhausted media could serve to concentrate cells (Jäger, 1996). Partial medium replacement is less costly and allows the retention of potential growth-promoting factors secreted by the cells (Doverskog et al., 2000; Ikonomou et al., 2004; Calles et al., 2006a; Calles et al., 2006b). Complete medium replacement, originally proposed in the early 90's (Lindsay and Betenbaugh, 1992), helps to obtain high production with Sf-9 cells (Table 1-6). Today's consensus seems to be that for large-scale applications, this solution is neither economical nor practical.

Therefore, the recovery of high productivities on a large scale is a challenge. Different strategies such as fed-batch schemes, perfusion operation and fixed-bed culture have been developed to provide nutrients to the cells without complete medium changeover. As in case of medium partial replacement, fed-batch, perfusion and fixed-bed prevent the removal of the autocrine factors produced by the cells during the growth phase, which are beneficial for growth and infection (Calles et al., 2006a). The majority of these feeding strategies has been applied to Sf cell lines, and fewer to Tni cell lines. Only the most recent studies address High-Five cells.

1.6.2. CONTINUOUS CULTURE

Continuous culture (CSTR) involves the replacement of culture medium without a cell retention system. It has not been widely used for production of baculovirus or r-protein with insect cells because the continuous withdrawal of the culture causes the dilution of cells and product (Drews et al., 1995). Moreover, the two steps of production (growth and infection) are not easily implemented in a continuous mode in the same reactor due to the transient nature of BEVS. Thus, continuous operating in a cascade of reactor has been proposed. It generally consists in two (or more) continuous serial stirred-tanks where the first is used for cell growth and the next for infection (Zhang et al., 1993) (Table 1-6). Unfortunately, its adverse affect is the accumulation of defective virus (van Lier et al., 1996).

On the contrary, perfusion, a continuous culture equipped with a cell retention system, is more widely used with insect cells because it allows to increase the cell density (Chico and Jäger, 2000). Even though many cell retention systems exist, mainly membrane-based retention systems (internal or external) are used in IC-BEVS perfusion.

Although there are few reports on lepidopteran cells cultivated in perfusion, all of them report very high cell densities and productions levels (Table 1-6). When cultivated in perfusion, Sf cells have been found to reach very high density, for instance $55x10^6$ Sf-21 cells/ml, which is the highest insect cell density reported in perfusion culture (Deutschmann and Jäger, 1994a), while the highest density reported with Sf-9 cells was $30x10^6$ cells/ml (Cavegn and Bernard, 1992; Cavegn et al., 1992) and $15.5x10^6$ cells/ml with High-Five cells/ml (Ikonomou, 2002). The highest production reported, in perfusion mode, using insect cells was ca. 500 mg/ml with High-Five cells (Chico and Jäger, 2000).

Perfusion has the advantage of continuously removing toxic by-products, of increasing cell density and of reducing the residence time of secreted r-protein in the reactor, hence limiting r-protein degradation. It also leads to high reactor-base (volumetric) productivity and allows efficient pH and DO control. Unfortunately, high perfusion rates

are costly in medium and dilute the product (Ozturk, 1996). Further, retention systems are often expensive, difficult to apply and can cause damage to the cells (Castilho and Medronho, 2002) whose membranes may have already been compromised by the baculovirus infection. In long-term perfusion, cell aggregation may appear (Ozturk, 1996) and DIP concentration tends to increase with the number of replication cycles (Krell, 1996).

Moreover, the use of high MOI in perfusion can be problematic, especially when only low-titer viral stocks are available (Jäger, 1996). As a rule, perfusion is advantageous in continuous culture with stable animal cell lines expressing a secreted protein. In contrast, IC-BEVS is a transient system producing protein for a short time coupled with cell lysis at the end of the infection (Rhodes, 1996). Thus, the perfusion culture of insect cells is better suited for the growth phase than for the infection phase.

1.6.3. MICROCARRIER CULTURE

The use of microcarriers (porous or non porous) in suspension in different bioreactor types (classical stirred-tank or airlift) is a good way to increase the available growth area for anchorage-dependent cells per unit of available bioreactor volume (Looby and Griffiths, 1990; Reuveny, 1990; Rundstadler et al., 1990; Griffiths and Looby, 1991; Kong et al., 1999). Lazar (1991) was the first to use this technology with insect cells for the large scale production of mosquito cells. Agathos et al. (1990) obtained promising results from the scale-up point of view with mosquito cells. However, few reports exist on the use of microcarriers for lepidopteran cell lines, perhaps because they are essentially attachment-independent (Archambault et al., 1994). Chung et al. (1993) cultivated Tni cells immobilized on collagen-coated microcarriers in a split-flow airlift bioreactor. Most recently, a comparison of immobilization of High-Five and Sf-9 cells on several types of microcarriers was made by Ikonomou et al. (2002).

Although microcarrier cultivation is relatively easy to apply, allowing to cultivate the scale-up of adherent cells and increasing considerably the volumetric production, the anchorage-independent nature of most insect cell lines and the one-shot, lytic nature of the BEVS causes many problems such as asynchronous infections and the establishment of nutrient gradients in the carriers (Ikonomou et al., 2002).

Moreover damage to the cells can come from high turbulence and microcarriers interactions in vigorously agitated cultures, whereas the potential formation of cell aggregate bridges between carriers can make the cultivation more heterogeneous (Drugmand et al., 2001). Finally, cell encapsulation, widely studied in the 90's, does not seem to have much of a future for industrial applications due to the heterogeneity of the culture and difficulties in harvesting of r-protein and in scale-up (Wu and Goosen, 1996).

1.6.4. FED-BATCH

Fed-batch operation involves essentially the intermittent addition of nutrients to a batch culture. It is an alternative that allows to increase the cell density and productivity without medium depletion. It has several advantages such as the fact that it is easier and cheaper to implement than perfusion and avoids exposure of the cells to high shear stress in a cell retention device (Table 1-7).

However, it requires knowledge of cell physiological requirements. Moreover, in fed-batch mode, accumulation of by-products may be considerable. Nevertheless the low sensitivity of insect cells to by-products accumulation, to osmolarity increase and to pH variation has made them a better candidate for fed-batch processing than mammalian cells.

Generally, fed-batch cultivation has addressed the increase of production and/or cell density but some articles have addressed the use of fed-batch to study insect cell metabolism (Nguyen et al., 1993; Öhman et al., 1995; Jang et al., 2000). Fed-batch cultivation of insect cells has been studied intensively in the last several years, especially in an effort to increase the cell density and/or production level using serum-free media and various feeding strategies (Table 1-6). Bédart et al. (1994) demonstrated, by a factorial experiment, that Sf cells needed a single pulse addition of Yeastolate and amino-acid mixture as supplement. However, the use of a richer supplement consisting of glucose, tyrosine, vitamins, iron and trace-metal solution lead to very high densities ($30x10^6$ cells/ml) (Bédard et al., 1997).

Table 1-7: Major benefits and drawbacks of fixed-bed cultivation of non-adherent cells

Benefits
Increased cell density[a,b,c]
Increased volumetric productivity[b,c]
Easy to apply on large scale[c]
Low-cost excessive technology[c]
No shear stress from a cell retention system
Drawbacks
Growth rate affected by cell density[c]
Productivity affected by cell density[d]
Accumulation of by-products[c]
More difficult to control than a batch (pH, DO,etc.)[b]
Knowledge of cell physiological requirements needed

a: Bédart et al., 1994; b: Elias et al., 2000; c: Ikonomou et al., 2003; d: Chico and Jäger, 2000.

In 2000, Elias et al. found that continuous addition is better than single pulse addition. The highest cell density reported so far with lepidopteran cells in fed-batch was $52x10^6$ cells/ml (Elias et al., 2000). However, the decrease of specific productivity associated with the increase of cell density ("cell density effect") resulted in unsuccessful infection at such a cell density. The highest reported Sf-9 cell densities at infection sustaining high production were $11.5x10^6$ cells/ml (Bédart et al. 1997), 12 $x10^6$ cells/ml (Chan et al., 1998) and $17x10^6$ (Elias et al., 2000) cells/ml.

Concerning High-Five cells, few data exist on their fed-batch cultivation. In 1998, Wu et al. attained $4x10^6$ cells/ml and infected cells in IPL-41 medium with 10 % FBS. Wang and Doong (2000) developed a serum-free fed-batch strategy based on the pH as indicator of a metabolic switch linked to the cells's requirements for glucose and Gln. Most recently, we have developed (This thesis, Chapter 5) a feeding strategy based on an understanding of the High-Five cells' needs during infection. We developed a feeding strategy that has resulted in high productivities consisting in pulses of media supplemented with glucose, glutamine, Primatone and Yeastolate whose formulations evolve as a function of cellular requirements. In 2003, Marteijn et al. proposed a genetic algorithm to optimise the culture media in a fed-batch of a *Helicoverpa zea* cell line.

1.6.5. Fixed-bed

Cultivation of insect cells in fixed-bed, also named packed-bed reactors, consists in the use of a immobilization matrix composed of non-oven fibres, foams or macroporous micro-supports compactly loaded into a retention basket within the reactor (Goosen, 1993). The main advantage of the fixed-bed is its ability to retain the cells allowing to perfuse the reactor with low shear stress (Table 1-8). Moreover, it allows to reach high cell densities in small bioreactors with high productivity (Reuveny, 1990). However, the density of the immobilized cells in the bed is difficult to estimate. Moreover, the distribution of cells inside the bed can be heterogeneous and several problems associated with gas exchange and metabolite distribution exist inside the bed (Rundstadler et al., 1990). In addition, the packed cells must remain viable and productive for prolonged periods of time during of the culture (Meuwly et al., in press).

Up to now, the majority of insect cell immobilization studies have been done on encapsulation (Pech and Strand, 1996) or with microcarriers (Ikonomou et al., 2002) and there are only few reports on insect cell cultivation in packed-bed reactors (Chiou et al., 1991; Kompier et al., 1991; Chiou et al., 1998a; Chiou et al., 1998b) (Table 1-9). These packed-bed studies were mainly done with Sf cells immobilized on microdisks or foam.

However, High-Five cells seem to also reach high cell densities and high product levels when trapped inside fixed-bed matrices.

Table 1-8: Major benefits and drawbacks of fixed-bed cultivation of non-adherent insect cells.

Benefits
Increased cell density [a,b,c,d]
Mild agitation and low shear stress [c,d]
Ease of perfusion cultivation [a,c,d]
Ease of product harvest [c,d]
Ease of scale-up [a,b,c]
Low bioreactor volume [a,b,c,d]
Drawbacks
Decreased growth rate due to support confinement and high cell density [d,e]
Difficulty of estimation of the immobilized cell density[c]
Heterogeneity of cultivation [a,b,c,d]
Oxygen and metabolite gradients [a,b,c,d]
Possible adsorption of the product on the support [e]

a: Rundstadler et al., 1990; b: Lazar, 1991; c: Archambault et al., 1994; d: Wu et al., 1996.

Table 1-9: Fixed-bed cultivation strategies for recombinant protein production in the BEVS.

Cell line	Fixed-bed strategy	Medium	X_{max} (10^6 cells/ml) (growth)	Recombinant protein / virus	Maximal Production	Reference
Sf-9	Packed-bed with (R_{100}) fibrous matrix	IPL-41	2.5	papain	75 % of supension culture	Archambault et al., 1994
	BelloCell reactor (BioNOC II ™ microcarriers)	TNM-FH with 10 % FBS	14	virus	$1.1x10^{15}$ pfu/ml	Hu et al., 2003
	Packed-bed with Dacron™ fibre	YPR	23	-	-	This thesis, Chapter 6
	BelloCell reactor (BioNOC II ™ microcarriers)	TNM-FH with 10 % FBS	13	EGFP	46.3 U/ml	Lu et al., 2005
	BelloCell reactor (BioNOC II ™ microcarriers)	YPR	4.2	-	-	This thesis, Chapter 6
Sf-21	Packed-bed with Fibra-Cel™ microcarriers	TNM-FH with 10 % FBS	6	β-gal virus	360 µg/ml $2.3x10^8$	Kompier et al., 1991
	Packed-bed with cellulose and polyurethne foam (PFU)	TNM-FH with 10 % FBS	5.2 with cellulose 4.3 with PFU	IL-5	$4x10^5$ U/ml	Chiou et al., 1998a and 1998b
High-Five	Packed-bed with BioNOC II™, Fibra-Cel™ microcarriers or Dacron™ fibre	YPR	24 with BioNOC II 15 with Fibra-Cel 10 with Dacron fibre	β-gal 1235 U/ml with BioNOC II 1115 U/ml with Fibra-Cel		This thesis, Chapter 6
	BelloCell reactor (BioNOC II™ microcarriers)	YPR	5.6	-	-	This thesis, Chapter 6

In fixed-bed cultivation, the choice of the support is the most important technical option. It must satisfy economical criteria (cost, quantity required, etc.) and its physical-chemical properties (chemical composition of the inner core and of the surface, surface charge, porosity, tortuosity, etc.) must respond to considerations of cultivation and production (seeding efficiency, immobilisation efficiency, growth rate, volumetric and specific production of the immobilized cells and non-adhesion of r-protein to the support) (Rundstadler et al., 1990; Archambault et al., 1994; Wu and Goosen, 1996).

1.7. INSECT CELL TECHNOLOGY: APPLICATIONS, MARKET AND PRODUCTS

The initial field of application of insect cell technology was the production of baculoviruses as insecticides, well developed since the 70's. The production of r-proteins in insect cells is more recent. Lately, the use of baculoviruses as gene delivery vehicles opens exciting new applications for gene therapy. Today, insect cells in culture are mainly used to produce r-proteins in the areas of research and biomedicine and continue to be used for the production of biopesticides. In addition, nowadays, lots of products are available for the construction of recombinant baculovirus and for insect cell cultivation. These enabling products are reviewed at the end of this section.

1.7.1. INSECTICIDES

The cost of damages inflicted by pest insects and their control is worth US$ 1.25 billon each year in the United Stated alone (FAO: Food and Agriculture Organization of the United Nation). The total world market of all insecticides is estimated at ca. US$ 8 billon (in 2005) and at US$ 150 million for bioinsecticides, of which baculoviruses account for US$ 10 million of sales. The first use of baculovirus in agriculture was in 1892, although they had been intensively studied as wild-type viruses since 1975 (Belisle et al., 1991; Bishop, 1994; Thiem, 1997). Today, more than 40 wild-type baculoviruses are used as biopesticides against damaging lepidopteran insects (moths, butterflies, larvae, etc.) that damage cotton, corn, tobacco, vegetables, grapes and many more crops (Cory, 2000; Palomares et al., 2006). Since 1988, many recombinant baculoviruses have also been used. They represent today the majority of the market. It was estimated that ca. 7 million of hectares of crops were treated with baculoviruses (FAO). These baculoviruses sold as biopesticides could be conditioned in form of liquids, powders and sprays. Treating 1 ha requires between 10^{12} to 10^{13} viruses (Palomares et al., 2006). The cost of treatment, which was very expensive in the 80's, is today around US$ 0.5/hectare (Palomares et al., 2006; FAO). At this cost, viral products are competitive with chemical insecticides. Although public safety concerns about genetically engineered products must be addressed whenever this issue is raised, these baculoviruses are, objectively, more health-care friendly and environmentally benign than chemical insecticides (Bishop, 1994; Maeda, 1995). The latter can accumulate in environment, can be dangerous for human and animal health and higher quantities of them are required per hectare, while baculoviruses are safe and lower amounts are needed to spread per hectare. They have no known adverse effects on non-target organisms (Maeda, 1995). However, although no deleterious effects of baculoviruses as

biopesticides on human health have been observed, no strict demonstration of complete safety has been offered so far (Bonning and Hammock, 1996; Palomares et al., 2006). They are specific against a narrow spectrum of pest insects (Maeda, 1995). Until now, insects show a lower tendency to develop resistance against baculovirus than against chemical insecticides (Fuxa, 1993). Nevertheless, this environmentally friendly advantage could become harmful when crops are infested by various several different pest insect species.

The baculovirus pesticides need to correctly identify the target pest due to their high specificities of and usually these biopesticides are not preventive. Contrary to chemicals, the insecticidal activity of baculoviruses is more susceptible to adverse environmental conditions (rain, sun, temperature, dryness, etc.) and to fertilizers. Wild-type baculoviruses do not kill pest insects as rapidly as chemical insecticides. Their lethality can be delayed between 3 days and 3 weeks. During this time, damages to crop can continue (Maeda, 1995).

Since the early 90's, several recombinant baculoviruses were developed to express various toxins from bacteria, mites, scorpions and wasps; juvenile enzymes; hormones and viral enhancing proteins under the *polh*, *p10* or other promoters. These genetically engineered baculoviruses are more effective with a higher lethal yield on pest insects (by up to 95%) and with a more rapid effect (3 to 8 days) (Bonning and Hammock, 1996; Palomares et al., 2006). On the other hand, some baculoviruses have been modified to increase their action spectrum to more insect species, but they must be used carefully because they could infect non-target beneficial species. In recombinant baculoviruses, the *polh* gene is deleted; hence they do not express polyhedrin and are non-occluded. They are less protected than wild-type baculoviruses from ultraviolet-B rays (from sunlight) causing DNA damage (O'Reilly et al., 1992). They are unable to initiate an in vivo secondary infection cycle and, on this account too, they are more environmentally friendly (Lawrence, 1996).

Usually, wild-type baculoviruses intended as pesticides are produced in vivo in larvae while recombinant baculoviruses are generally produced in vitro. In vivo production requires breeding of caterpillars from eggs in humidified containers at 25-27°C containing ca. 15 larvae where their diet is controlled (O'Reilly et al., 1992; Thiem, 1997). Infections are done by spraying occluded viruses onto the larvae or by addition them into the diet. Viruses are harvested after the second virus cycle from larval hemolymph and their yield is ca. 10^9viruses/caterpillar (Hughes and Wood, 1996). Non-occluded virus (*polh* gene deleted) cannot be produced in vivo (Maeda, 1995; Lawrence, 1996). In vitro production of baculoviruses is more expensive than in vitro production and requires qualified staff, but it

is more practical for quality control, especially in the production of recombinant viruses, as long as the market size does not exceed 1 million hectares a year (Christian and Scotti, 1996; Palomares et al., 2006).

1.7.2. RECOMBINANT PROTEINS

Today, in addition to the production of pesticides, the IC-BEVS technology is widely used for the production of r-proteins for various commercial, biomedical and medical research applications and for production of diagnostic reagents. More than 500 genes have been expressed in IC-BEVS and this system is chosen in almost 50 % of the scientific papers reporting a higher eukaryotic protein expression (Palomares et al., 2006). Theses sectors of applications need great amounts of r-proteins. Biological activity and similarity to native proteins represent great potential for the production in insect cells.

Although the majority of r-proteins is produced in vitro using the IC-BEVS, these proteins may be also produced in vivo in larvae, or in stable (constitutively engineered) insect cells lines (Luckow, 1991; O'Reilly et al., 1992).

The yield of production of r-protein in larvae could represent 50 % of biomass protein of the larvae (Cha et al., 1999; Pham et al., 1999). However, in vivo production needs experience in growing and maintaining larvae. It also needs efficient purification steps of the r-protein and is only used when the amount produced is more important than the purity required (in diagnostic applications or in the research area). This technology uses mainly silkworm larvae (*Bombyx mori*) infected with BmNPV virus but can also use larvae of the cabbage looper *Trichoplusia ni* infected with AcMNPV. This approach is more widespread in Asia where silkworm cultures are well developed (Kost et al., 2005).

Concerning stable cell lines (constitutively engineered), insect cells are able to produce in continuous mode up to several mg/l, sometimes with higher secreted protein yield than the BEVS and occasionally without the risk of protein degradation due to lysis in the BEVS (Jarvis, 1991; Fallon, 1996; Farell et al., 1998). Despite these advantages, the establishment of stable insect cell lines expressing r-proteins in a non-lytic system does not seem to have much of a future in industrial application, because cells generally produce less protein, the time for establishment of a stable cell line is longer and more expensive than BEVS and, overall, this approach is not really less expensive than the production in mammalian cells. The latter are preferred by industry because they are more under control, there is more known on their bioprocess behavior, and they possess the capacity for post-translational modifications nearest to human proteins (McCaroll and King, 1997).

1.7.3. THERAPEUTICS

The efficacy, safety and cost issues appear to indicate that for some products the production of recombinant protein or viral drugs using IC-BEVS will be useful. The BEVS constitutes a good system the production of vaccine subunits by expressing antigen proteins or by production of virus-like particles (VLP) as vaccines (Schmidt and Hoffman, 2003; Schmidt, 2004). A VLP is a virus identical to the native virus expressing surface protein from one or more different pathogen viruses, but without their genetic material. This approach is particularly attractive for producing vaccines against viruses whose viral replication is impossible with a cell culture-based technology, such as human papilloma virus and hepatitis virus. Today, the presence of glycosylation a bit different from mammalian cells is still considered as a disadvantage for insect cell based production of human therapeutics. However, for vaccine production, the lack of human-like glycosylation of the antigen is less important, and it can even enhance the immune response to the vaccine (Schmidt and Hoffman, 2003).

The first therapeutic produced using IC-BEVS was a veterinary vaccine against the swine fever virus, approved in 2000 (Intervet International, Boxmeer, The Netherlands). Today, four commercialized veterinary drugs are made using IC-BEVS (Table 1-10) (Walsh, 2005). The market of IC-BEVS is worth ca. 1 % of the biopharmaceutical market (US$ 478 billion in 2004) but will probably increase in the next years (Cox, 2004).

Table 1-10: Recombinant veterinary drugs produced using the Insect Cell-Baculovirus system approved in the EU.

Product	Type	Company	Therapeutic indication	Approved
Porcilis pesti	Vaccine	Intervet International (Boxmeer, The Netherlands)	Immunization of pigs against classic swine fewer virus	2000
Bayovac	Vaccine	Bayer (Leverkusen, Germany)	Immunization of pigs against classic swine fever virus	2001
Virbagen Omega	Interferon	Virbac (Carros, France)	Treatment of dogs infected with canine parvovirosis and cats infected with feline retrovirosis	2001
Advasure	Vaccine	Pfizer (New York, NY)	Immunization of pigs against classic swine fever virus	2004

Concerning human drugs, the fear of new technology and the cost of drug development has been one of the brakes on their development using insect cells (between US$500 and 800 million for the development of a human vaccine). However, the benefits of the BEVS and the recent approval of veterinary products by the American Food and Drug Administration (FDA), the European Medicines Agency (EMEA), the Australian Therapeutic Goods Administration (TGA), The Japanese Ministry of Health, Labour and Welfare (MHCW) and the Public Health Agency of Canada have allowed pharmaceutical manufacturers to develop new human therapeutic products using insect cells.

Nowadays, many therapeutic vaccines and drugs using IC-BEVS technology are under development ("in the pipeline"). Amongst vaccines, Protein Sciences (Meriden, CT) has begun the phase III clinical trials of an Influenza type A vaccine, the most common type of virus causing human flu. They have also a vaccine program against HIV-AIDS based on a similar approach. They have developed an avian vaccine against bird flu (Influenza H5) in vivo with caterpillars. The company has, in phase II clinical trials, an epidemiologic human vaccine produced in vitro by IC-BEVS, which is usable in case of a pandemic of avian flu virus and transmission to humans. Concerning cancer vaccines, Dendreon Coorporation (Seattle, WA) and GlaxoSmithKline (Rixensart, Belgium) have, respectively, a prostate cancer vaccine candidate and a human papilloma virus vaccine candidate in phase III clinical trials. MedImmune Inc (Gaithersburg, MD) has just licenced a human papilloma virus vaccine and will commercialize it in 2006 or 2007. Concerning hepatitis, GlaxoSmithKline has an hepatitis E vaccine candidate in development using insect cells. Wyeth (Madison, NJ) has development programs including production from insect cells of a HIV, a malaria and a needle-free flu vaccine. As far as therapeutic drugs are concerned, Protein Sciences will soon bring to human testing an erythropoietin produced with IC-BEVS. Amgen (Thousand Oaks, CA) and Sanofi-Pasteur (Paris, France) have programs of vaccine and therapeutic drug development using insect cells that are in preclinical phase. Human Genome Sciences (Rockville, MD) has under development some therapeutics using insect cells that are in the pre-clinical and clinical phases. Bayer (Leverkusen, Germany), Intervet International, Pfizer (New York, NY) and Vibrac (Carros, France) are supposed to continue to develop veterinary products using IC-BEVS. Pfizer and Bayer are not disclosing any information regarding the development of human therapeutic using IC-BEVS. However their choices to produce veterinary vaccines using this technology indicate an interest in the IC-BEVS platform and perhaps a future development of human therapeutics. Nowadays, most other human and animal health companies involved in the production of r-proteins as therapeutics are using mammalian cells: Baxter (Deerfield, IL,

U.S.A), Eli Lilly (Indianapolis, IN), Genentech (South San Francisco, CA, U.S.A.), Genzyme (Cambridge, MA), Hoffmann-La Roche (Basel, Switzerland), Merial (Saint-Priest, France) do not officially communicate an interest in BEVS technology. Other vaccine projects using IC-BEVS are under development by several companies and institutes: veterinary vaccines in preclinical phase against the foot-and-mouth-disease and the canine leishmaniasis; the development of a human vaccine candidate against HIV-AIDS, malaria, hepatitis A, B, C and E, human hookworm disease, human parvovirus and human polyomavirus (WHO: World Heath Organisation). Various therapeutic drugs are currently under investigation, e.g. antistasin, erythropoietin, interleukins, tPA, glucocerebrosidase, etc. (Palomares and Ramirez, 1998).

Despite the growing interest in insect cell culture and technology, it seems clear that several large pharmaceutical companies have opted, at least for the time being, not to develop the production of r-proteins using the BEVS, due to key patents controlling insect cell technology (over than 5000) such as patents on media, cell lines, applications, vector constructions, etc. The moment the major such patents will expire and the first product for human therapy approved, a lot of people will probably start using the IC-BEVS for commercial applications, including human health.

As already shown, the use of baculovirus and insect cell technology offers many opportunities for the production of vaccines. However, some companies prefer to use mammalian cells that have better glycosylation than insect cells (Klenk, 1996; Breitbatch and Jarvis, 2001; Marchal et al., 2001). Or course, the recent studies on insect cells expressing “human like” glycosylation patterns by eliminating “insect-like” glycosylation and replacing the insect genes with genes encoding for enzymes that mimic mammalian/human glycoform synthesis, could change this (Jarvis, 2003; Tomiya et al., 2003). Insect cells stably transformed with mammalian glycosyl transferases (Hollister et al., 1998; Hollister and Jarvis, 2001; Hollister et al., 2002) have given rise to SfSWT-3 cell line (a transgenic derivative of Sf9 cell line) (Aumiller et al., 2003), which can form monosialylated biantennary complex N-glycans in serum-free medium supplemented with N-acetylmannosamine. Its parental cell line, SfSWT-1 is commercially available by Invitrogen (Carlsbad, CA) under the name “Mimic cells” and can produce biantennary N-glycans like SFSWT-3, but requires a serum-supplemented medium. The establishment of insect cell line expressing proteins with “human-like” glycosylation is a challenge to improve the quality of vaccines and therapeutics produced in insect cells (Jarvis, 2003; Kost et al., 2005).

The three dominant insect cell lines in industrial processes are the Sf-9, Sf-21 and High-Five. They are able to grow in serum-free media on large scale. At present, industrial processes use mainly Sf-9 cells because during their development in the 90's, the Sf-9 cells were better known and considered most productive. Today, High-Five cells are proving more efficient for large-scale cultivation, are regarded as the most productive insect cell line and seem to be more and more popular in new process development. Finally, although High-Five cells are known to produce more r-protein but fewer viruses on a per cell basis, than Sf cell lines, they seem present great industrial interest to produce VLP.

Concerning the regulations around the rise of insect cell for therapeutic application, same safety guidance from experience with other animal cells must be followed (van Lier, 1996). Cell lines must be free from mycoplasma, retrovirus and adventitious virus but they must be also free of entomoplasma, mesoplasma and spiroplasma viruses known to potentially contaminate invertebrate cell lines (Galbraith, 2002; Galbraith, 2005). The absence of residual protein, RNA and DNA must be checked in the final products and abnormal toxicity and pyrogenicity must be controlled (Bernard et al., 1996).

1.7.4. Other applications of baculovirus

In the middle of the 90's, another application of insect cells became the use of baculovirus display for the screening of peptides or proteins. Various strategies have been developed for displaying heterologous peptides or proteins on the surface of baculovirus particles by attaching peptides or proteins to the surface glycoprotein gp64 of the AcMNPV. This phosphoglycoprotein, present as a homo-trimer on the surface of virons is the major coat protein of the virus. Its abundance, its transcription, which is controlled by a strong promoter, and the feasibility of modifing its N-terminus make the gp64 a good candidate for generating a library of peptides or proteins expressed on the viral surface. The baculovirus particles can provide a unique scaffold for assembly and enrichment of functional membrane bound glycoprotein complexes in eukaryotic cells (Grabherr and Ernst, 2001; Kost et al., 2005). However, the baculovirus display seems to generate less abundant libraries compared to prokaryotic virus display technologies (e.g. phage display). Up to now, the baculovirus display has allowed the screening of antibodies and drugs, the study of viral surface interactions with host cells, the enhancement of baculovirus surface-mediated gene delivery into mammalian cells, etc. (Grabherr et al., 1997; Grabherr and Ernst, 2001).

Another novel application of IC-BEVS appeared at the end of the 90's, when baculoviruses started being studied as gene delivery vehicles for r-protein production in mammalian cells (Condreay and Kost, 2003) and as vectors for gene therapy (van Loo et

al., 2001). Baculoviruses are able to deliver genes into various non-dividing vertebrate cells (human, monkey, mouse, porcine cells) without triggering the virus replication and lysis observed when viruses infect insect cells. However, the entry mechanism and fate of baculoviruses in mammalian cells are not well understood. Baculoviruses can be internalized by mammalian cells but their promoters remain silent. Although the *polh* promoter has been demonstrated to be inactive in mammalian cells, other promoters could be used for expression of r-protein in mammalian cells or for gene transfer (Palomares et al., 2006). Moreover, these vector candidates are non pathogenic for humans, pre-existing immunity in humans is not likely and they do not need helper virus (Stacey and Posee, 1996). Baculoviruses are known to be inactivated by the immune system, but direct injection of virus in tissues have allowed to deliver genes into the brain or muscle of mammals (Sarkis et al., 2000; Pieroni et al., 2001). Many publications focused on the use of recombinant baculoviruses to direct gene delivery in various applications, such as heterologous gene expression in mammalian cells, genomics research, pharmaceutical screening assay or in vivo gene therapy. However, only the direct gene introduction into mammalian cells to establish new stable cell lines able to produce r-protein seems to have an obvious industrial application. The use of baculoviruses as gene transfer vehicle candidates for gene therapy is hopeful but more background knowledge is needed to see if this technology could have realistic applications in the future.

1.7.5. ENABLING PRODUCTS

The increase of insect cell applications has lead to the arrival on the market of various products specifically developed for insect cell technologies. The last comprehensive reviews of these products date from the protocol book of O'Reilly and co-workers (1992), the protocol book of Richardson (1995) and the article of Merrington et al. (1997).

There is a considerable number of available insect cell lines and most of them are sold already adapted to suspension culture. The European Collection of Animal Cell Cultures (ECACC) and the American Type Culture Collection (ATCC) sell most of them adapted to serum-free suspension culture. However, the following companies offer insect cell lines pre-adapted to cultivation reaching high densities: BD Biosciences Pharmingen (Franklin Lakes, NJ): Sf-9, Sf-21 cells lines adapted to SFM; Gentaur (Brussels, Belgium): Tni, Sf-9, Sf-21 cell lines; Invitrogen (Carlsbad, CA): Sf-9, Sf-21, "Mimic cells", High-Five, D.Mel-2 (cell line from *Drosophila melanogaster*) cell lines adapted to SFM; Merck Biosciences Novagen (Darmstadt, Germany) : Sf-9 cell line; Riken Bioresource Center Cell Bank (Ibaraki, Japan): Bm and D.Mel cell lines.

Nonetheless, the use of a patented cell line, such as the High-Five cells, for commercial application requires the payment of royalties.

Table 1-11: Enabling Products for IC-BEVS Applications

Product	Company	Features
Baculoviurs expression vector systems sold as kits		
Backpak6™	AB Vector (San Diego, CA)	Modified baculovirus vector In vitro recombination No plaque selection
BacMagic™	Merck Biosciences Novagen (Darmstadt, Germany)	Modified baculovirus vector Uses polyhedrin promoter In vitro recombination No plaque selection
Bac-N-Blue™	Invitrogen (Carlsbad, CA)	Modified baculovirus containing *LacZ* gene In vitro recombination Simplified selection, plaques are blue
BacTen™ Baculovirus	Q-BioGene (Irvine, CA)	Modified baculovirus vector In vitro recombination No plaque selection
Bac-to-Bac™	Invitrogen	Modified baculovirus vector Use of polyhedrin promoter Recombination in *E. coli* Plaque selection
BaculoDirect™	Invitrogen	Modified baculovirus vector In vitro recombination No plaque selection
BaculoGold™	AB vector (San Diego, CA) and BD Sciences (Franklin Lakes, NJ)	Modified baculovirus vector containing lethal deletion and GFP gene In vitro recombination No plaque selection Infected cells are fluorescent
BaculoKIT Gold™	Protein Sciences (Meriden, CT)	Modified baculovirus vector In vitro recombination Simplified selection
BaculoKIT™	Protein Sciences	Modified baculovirus vector In vitro recombination Simplified selection
Baculovirus Expression Booster™	Orbigen (San Diego, CA)	Recombinant baculovirus expressing unique set of eukaryotic chaperones In vitro recombination Simplified selection 10 different kits
Baculovirus IKM™ technology	BlueSkyBiotech (Worcester, MA)	Modified baculovirus vector In vitro recombination Simplified selection

Product	Company	Features
BacVector™	Merck Biosciences Novagen (Darmstadt, Germany)	Modified baculovirus vector containing *LacZ* gene Use of polyhedrin promoter In vitro recombination No plaque selection 3 different kits
EasyXpress™	Qiagen (Oslo, Norway)	Modified vector, allows expression into *E. coli*, baculovirus-infected insect cells and mammalian cells Recombination in *E. coli* Simplified selection
FlashBAC™	Oxford Expression Technologies (Oxford, UK)	Modified baculovirus specified for high production in High-Five of Sf-9 In vitro recombination No plaque selection
pFastBac™	Invitrogen	Modified baculovirus vector Allows cloning of two proteins in the same baculovirus In vitro recombination No plaque selection
ProEasy™	AB vector	Modified baculovirus vector; Uses polyhedrin promoter In vitro recombination No plaque selection
ProFold™	AB vector	Baculoviurs vector containing chaperon and GFP gene optimized for cytoplamic, membrane or secreted protein In vitro recombination Simplified selection 4 different kits
Baculovirus vectors		
Baculovirus transfer vector	Gentaur (Brussels, Belgium)	Modified baculovirus vector
Cytotools™ recombinant baculoviruses	PerkinElmer (Boston, MA)	Modified baculovirus developed for the expression of G Protein-Coupled Receptor (GPCR) tools
pAc-l-CH	Progen (Heidelberg, Germany)	Baculovirus casette vector for expression of human, humanized or chimeric IgG(gg) in insect cells
pAc-l-FC	US Biological (Swampscott, MA)	Baculovirus cassette vector for expression of human, humanized or chimeric IgG(gg) in insect cell
pBAC™- E.coli Ek/LIC vector	Merck Biosciences Novagen (Darmstadt, Germany)	Modified baculovirus vector, Use of polyhedrin promoter
pIEX™	Merck Biosciences Novagen (Darmstadt, Germany)	Modified baculovirus vector Use of IE1 (immediate early) promoter
pPBac and pMBac	Stratagene (La Jolla, CA)	Modified baculovirus vector

The baculovirus most commonly used for protein expression is AcNPV. Since the development of BEVS, the availability of various recombinant baculoviruses has vastly increased (Table 1-11). Today, most of them are based on the *polh* and *p10* promoter genes, but other available vectors use alternative promoters, some of them expressed earlier than *polh* and *p10*. The majority of the commercially available baculoviruses are linear vectors needing no or only few selection steps. Commercial vectors are often offered in kit form with sufficient DNA material so that there is no need of bacterial amplification. Their recombinations are usually done either in vitro by restriction enzymes, or in insect cells. They contain modifications designed for making them more efficient, more productive or capable of expressing specific proteins (e.g. chaperons) in addition to the foreign gene.

Although the establishment of stable insect cell lines has become important in recent years, no stable insect cell lines expressing a foreign protein is sold commercially. The insect cell lines expressing proteins allowing "humanized" glycosylation are often patented. However, 4 kits containing non-lytic viruses are sold to specifically express r-protein in insect cell lines (Table 1-11). Meanwhile, Invitrogen (Carlsbad, CA), AB vector (San Diego, CA) and Protein Sciences are developing new innovative vectors to establish stable insect cell line and for IC-BEVS. Concerning, the expression of protein into infected larvae, 2 viruses are specifically sold to obtain high efficiency with in vivo infection (Table 1-12).

Today, more than 30 media are proposed for the cultivation of insect culture (Table 1-5). The media in the public domain are offered by lot of companies (Agathos, in press). Several companies sell proprietary media specifically developed for the cultivation of insect cells on a large scale. The majority of these companies also offer services of custom-made medium production. Other media are expressly developed for virus transfection and amplification, usually for a specific baculovirus vector sold by the same company. Key features of all of these media are summarized in Table 1-5.

Concerning virus titration, the most current methods are the end-point dilution and plaque assay, but these are time-consuming and several companies offer more rapid techniques. Hyclone (Logan, UT) sells a kit containing the necessary ingredients for plaque assay, shorting the time of preparation (HyQ BEVS plaKit) while Clontech (Mountain View, CA), Expression Systems (Woodland, CA) and Merck Biosciences (Darmstadt, Germany) offer quick and efficient ELISA kits for titrating virus based on the detection of the gp64 viral capsid protein with a monoclonal antibody.

Table 1-12: Enabling products for establishment of stable insect cell lines and for production in larvae

Product	Company	Localisation	Vectors
Vectors for establishment of stable insect cell lines			
InsectSelect™	Invitrogen	Carlsbad, CA	Vector for non-lytic expression in Sf-9, Sf-21 and High-Five (lepidopteran) and S2 (dipetran) cells Sold as a kit
DES®	Invitrogen	Carlsbad, CA.	Vector for non-lytic expression in S2 cells Sold as a kit
pIEX™	Merck Biosciences	Darmstadt, Germany	Vectors designed for the establishment of stable insect cell lines
Sapphire™ Insect Transfection	Orbigen	San Diego, CA	Vectors designed for the establishment of stable insect cell lines Sold as a kit
Vectors for production of recombinant protein into insect larvae			
Entopath's Larval Express®	Entopath	Easton, PA	Vector for expression in insect larvae using baculovirus Sold as a kit
Insect-Direct™ system	Merck Biosciences	Darmstadt, Germany	Vector for expression in insect larvae without lytic virus Sold as a kit

Current estimations indicate that ca. 35 % of all biopharmaceutical companies outsource or subcontract raw materials or research and development (R&D) services (de Noronha Pissarra, 2004). The insect cell market has been adapting to this reality and more and more small companies subcontract services involving protein expression in insect cells for research or for other industries (Table 1-13). A wide variety of services are proposed, regarding viruses, such as the construction of the requisite recombinant baculoviruses, their amplification, purification and banking as well as adaptation of insect cells to a given medium and cell banking.

Some companies subcontract also the production and the purification of r-proteins in larvae or by in vitro cultivation. Some of them offer the production of a r-protein in GMP conditions for clinical studies from the construction of the vector, all the way to the production and purification of the product(s) in 1 to 3 months, at a scale from 1 L to 1000 L for some of them, for a price frame round US$ 150 to 400 per litre of culture, as a function of the required features of the products and decreasing with quantities. Other companies are specialized in the scale-up of insect cell culture under GMP conditions. These companies and their services are listed in Table 1-13. Companies offering the products and services on insect cell technology are worldwide, with a higher concentration in California and

Germany, while companies producing baculoviruses as insecticides are located in Asia, North and South America. The international companies working on the development of human vaccines and veterinary therapeutic using BEVS are located in Europe and in North America. In the future, the new applications of insect cell products as therapeutics are expect to increase considerably the number of companies in the insect cell market.

Data were obtained from the websites of these companies.
Sources of market analysis include, but are not limited to, the websites of the companies involved reports of the World Heath Organisation (WHO), the American Food and Drug Administration (FDA), the European Medicines Agency (EMEA), the Australian Therapeutic Goods Administration (TGA), the Japanese Ministry of Health, Labour and Welfare (MHCW), and the Public Health Agency of Canada.

Table 1-13: Commercial companies offering Insect Cell Baculovirus System services

Company	Localisation	Service
Abgent	San Diego, CA	Production and amplification of recombinant baculovirus
AB Vector	San Diego, CA	Production of modified baculovirus vectors for enhanced protein expression
AMS Biotechnology	Oxon, UK	Production and amplification of recombinant baculovirus, optimization of insect culture scale-up and production and purification of recombinant protein
Applied Viromics	Fremont, CA	Production and amplification of recombinant baculovirus
ATG Laboratories	Eden Prairie, MI	Production and amplification of recombinant baculovirus and production and purification of recombinant protein
BDP	Partway, CA	Optimization and scale-up of insect cell lines
BD Biosciences Pharmingen	Franklin Lakes, NJ, U.S.A.	Production and amplification of recombinant baculovirus
Biocarta	Wako, Japan	Production and amplification of recombinant baculovirus and production and purification of recombinant protein
BioExpress	Lebanon, NH	Production of customized antibodies in insect cell line
Biomed	Crescent, Singapore	Production and amplification of recombinant baculovirus, optimization of insect culture scale-up and production and purification of recombinant protein
Biomedicum	Helsinki, Finland	Optimization and scale-up of insect cell lines
Biomol GmbH	Hamburg, Germany	Production and amplification of recombinant baculovirus and production and purification of recombinant protein
BioSciences Research Associates	Boston, MA	Optimization and scaling-up of insect cell lines
BlueSkyBiotech	Worcester, MA	Production and amplification of recombinant baculovirus
Chesapeake PERL	Savage, MD	Production of recombinant protein and baculovirus in insect larvae
Clinisciences	Montrouge, France	Production and amplification of recombinant baculovirus and production and purification of recombinant protein
CytoMol	Sunnyvale, CA	Production and amplification of recombinant baculovirus, optimization of insect culture scale-up and production and purification of recombinant protein
Diarect	Freiburg, Germany	Production and amplification of recombinant baculovirus, optimization of insect culture scale-up
Gencust	Evry, France	Production and amplification of recombinant baculovirus, optimization of insect culture scale-up and production and purification of recombinant protein

Company	Localisation	Service
Genscript	Piscataway, NJ	Production, purification and amplification of recombinant baculovirus
Henogen	Charleroi, Belgium	Optimization and scale-up of insect cell lines
IBA GmbH	Göttingen, Germany	Production and amplification of recombinant baculovirus, optimization of insect culture scale-up and production and purification of recombinant protein
Imgenex	San Diego, CA	Production, purification and amplification of recombinant baculovirus
Invitrogen	Carlsbad, CA	Production and amplification of recombinant baculovirus
Kinnakeet Biotechnology	Richmond, VA	Production and amplification of recombinant baculovirus and production and purification of recombinant protein
MegaGenex Biosciences	San Diego, CA	Production and amplification of recombinant baculovirus
Orbigen	San Diego, CA	Production and amplification of recombinant baculovirus and production and purification of recombinant protein
Oxford Expression Technologies	Oxford, UK	Production and amplification of recombinant baculovirus, optimization of scaling-up of insect culture and production and purification of recombinant protein
Paragon Biosciences	Baltimore, MD	Production, purification and amplification of recombinant baculovirus
PerkinElmer	Boston, MA	Expression of proteins into BEVS and stable insect cell lines
Pharmalicensing	York, UK	Production and amplification of recombinant baculovirus, optimization of scaling-up of insect culture and production and purification of recombinant protein
Protein Sciences Corporation	Meriden, CT	Production and amplification of recombinant baculovirus, optimization of scaling-up of insect culture
Qiagen	Oslo, Norway	Production and amplification of recombinant baculovirus
Q-BioGene	Irvine, CA	Production and amplification of recombinant baculovirus
ReliaTech	Braunschweig, Germany	Production and amplification of recombinant baculovirus
Rockland Immunochemicals	Gilbertsville, PA	Production and amplification of recombinant baculovirus
Shangai Genomics	Shanghai, China	Production and amplification of recombinant baculovirus, optimization of scaling-up of insect culture and production and purification of recombinant protein
The Wistar Institute	Philadelphia, PA	Production and amplification of recombinant baculovirus, optimization of scaling-up of insect culture

Company	Localisation	Service
University of Cambridge, Department of Biochemistry (contract manufacturing facility)	Cambridge, UK	Production, purification and amplification of recombinant baculovirus
University of Minnesota, Recombinant Protein Expression Laboratory (contract manufacturing facility)	St. Paul, MN	Production and amplification of recombinant baculovirus, optimization of scaling-up of insect culture

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PART II: INSECT CELL PHYSIOLOGY AND METABOLISM

2 PART II: INSECT CELL PHYSIOLOGY AND METABOLISM

Chapter 2 : Effects of dextran on growth behavior of High-Five lepidopteran cells in agitated suspension cultures

2.1. INTRODUCTION

In the last two decades, the Insect Cell - Baculovirus Expression Vector System (BEVS) has become popular for producing many recombinant (glyco-)proteins (Agathos, 1996; Ikonomou et al., 2003). The relative simplicity of insect cell cultivation in serum-free media and the easy construction of a recombinant baculovirus vector have made the BEVS quite an effective transient expression system.

The cell line High-Five from *Trichoplusia ni*, tends to express more cytoplasmic and secreted glycosylated proteins than *Spodoptera frugiperda* cell lines. For instance, High-Five cells were shown to produce 7-fold more β-galactosidase and 26-fold more human secreted alkaline phosphatase (Wickham et al., 1992; Wickham and Nemerow, 1993). Unfortunately *Trichoplusia ni* cell lines are attachment-prone and generate, in suspension culture, large clumps in bioreactors (Saarinen et al., 1999) and generally form a large compact mass of cells, called "cell ring", on the inner walls of culture recipients at the air-liquid interface. The presence of large aggregates makes it occasionally difficult to deliver virus to the cells in the inner core of such clumps and may result in a decrease in volumetric production (Wood et al., 1982).

Therefore, to promote the great protein expression potential of *Trichoplusia ni* cells, several studies have addressed cell growth in suspension by increasing agitation speed or by adding surface active compounds. An increased agitation speed eliminates cell clumping and can lead to a selection of a single-cell population but may also cause cell damage (Chalmers, 1996). Bulky surface-active macromolecules like Pluronic F-68 and polyethylene glycol (PEG) have the potential to protect the cells from shear damage when increasing agitation speed to diminish cell clumping (Murhammer and Goochee, 1988). In order to reduce clumping of cells grown in suspension, special media with low concentration in calcium and magnesium ions are available (McGlinchey et al., 1992). Addition of the calcium chelating agent EDTA decreases animal cell aggregation but such a non-specific chelator can cause cell damage by changing media osmolarity (Prokop, 1991). Addition of negatively charged derivatized polysaccharides such as sulfated heparin (Taticek et al., 1997) or sulfated dextran (Dee et al., 1997a; Dee et al., 1997b; Shuler and Dee, 1998) have been shown to reduce cell clumping at low concentrations but they inhibit baculovirus binding to cells. Sulfated heparin decreases production even when the cells are transferred to polymer-free medium before infection (Taticek et al., 1997) and sulfated polymers (including sulfated dextran) inhibit infection at low multiplicity (MOI < 1) (Dee et al., 1997a; Dee et al., 1997b; Shuler and Dee, 1998). Neutral carbohydrate polysaccharides

like methylcellulose, heparin, cellulose, polyvinyl alcohol, etc. have a lower potential for decreasing cell clumping and are toxic at high concentrations for both insect and mammalian cells (Goldblum et al., 1990; Michaels et al., 1992; Taticek et al., 1997).

To prevent cell ring formation in shaken culture, a light silicone coat is commonly applied on the bioreactor or flask inner walls but without great success on High-Five cells. Moreover, polycarbonate or polystyrene culture flasks are treated to inhibit adsorption of cells (McGlinchey et al., 1992).

Despite its potential to break up cell aggregates and its previously proven capacity to protect animal cells against hydrodynamic shear (Goldblum et al., 1990; Croughan et al., 1988a and 1998b), dextran has not yet been studied to prevent insect cell clumping.

In animal cell culture, lower and intermediate molecular weight (MW) dextran (39 and 90 kDa) had no effect on the growth of anchorage-dependent human fibroblasts in culture at 1-3 % (w/v) concentrations and protected the cells against high shear stress conditions, whereas at higher concentration dextran was inhibitory to growth (Croughan et al., 1988b). Cultivated on microcarriers, the same cells suffered less damage upon addition of 2 to 5 % (w/v) dextran of 78 kDa. For hybridoma cells increasing concentrations [1-3 % (w/v)] of higher MW dextran (229 kDa) was found to cause commensurate decrease in growth rate under stirred conditions (Croughan et al., 1988a). For insect cells it was reported that the addition of 4.5 % of even higher MW dextran (467 kDa) increased their resistance to cell lysis upon laminar shear stress (Goldblum et al., 1990). Dextran is known to protect erythrocytes from a low-pH induced hemolysis (Cudd et al., 1989). It is also known to protect suspension cultivated cells against damage from sparging (Chattopadhyay et al., 1995; van der Pol et al., 1995). Dextrans of MW > 40 kDa at very high concentrations [10-20 % (w/v)] seems to protect hybridoma cells from sparging damages by increasing medium viscosity (van der Pol et al., 1995), whereas 79 kDa dextran weakly shields insect cells from intensive sparging at concentrations of 0.5 and 5 % (w/v) (Chattopadhyay et al., 1995).

As a non-ionic polymer, dextran has some flocculation-inducing properties as a function of its molecular weight. High-MW dextrans increase aggregation of erythrocytes (Knox et al., 1977). Lower MW dextran (40 kDa) decreases clumping and leads to disaggregation of red cell *rouleaux* since they are too small to effect bridging (Jan et al., 1982). Dextran also enhances the exclusion of cations in the medium from cell-to-cell double layers and can create steric hindrance to the close approach of neighbouring cell membranes (Aunins, 1989).

For cell culture applications, the toxicity of dextran depends on its MW and on the cell type but generally increases with the MW, the concentration and the presence of functional groups on the dextran backbone. Whereas neutral polysaccharides seem to be non-toxic at high concentration, negatively charged polysaccharides were found to be toxic at high concentration and positively charged ones were highly toxic to animal cells (Aunins and Wang, 1989).

Hence, we decided to study the influence of dextran on High-Five cells growth and infection, in bioreactor and in shake-flask culture since the latter represents a quick and efficient way of obtaining sufficient inoculum for further cultivation in bioreactors (Neutra et al., 1992). The effect of dextran on the cell membrane and recipient surface properties was analysed to gain an understanding of this interaction.

2.2. MATERIALS AND METHODS

2.2.1. INSECT CELLS AND CULTURE CONDITIONS

The insect cells lines High-Five and Sf-9 were a gift from The Laboratory of Virology (University of Wageningen, The Netherlands). A recombinant baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcMNPV) expressing human secreted placenta alkaline phosphatase (seAP) was obtained from H.A. Wood (Boyce Thompson Institute of Plant Research, Ithaca, NY)

Suspended cells were cultured in shake flasks of 125 and 250 ml (Corning, Acton, MA) at 27°C and 135 rpm with respectively 25 and 50 ml of medium, whereas adherent cells were cultivated in T-flasks of 75 cm^2 (Nunc, Roskilde, Denmark). High-Five cells were inoculated at 0.2 $x10^6$ and Sf-9 at 0.4$x10^6$ cells/ml. Cell growth was also studied in a Celligen-Plus™ bioreactor (New Brunswick Scientific, Edison, NJ) with a working volume of 1L (dissolved oxygen: 50 % of air saturation, 80 rpm). The glass bioreactor was siliconized with Sigmacote (Sigma, St-Louis, MO) to prevent cell adhesion in control experiments.

Cell growth was examined in Insect-Xpress™ medium (Cambrex, Walkersville, MD) and in a serum-free homemade medium developed in our laboratory, called YPR (Ikonomou et al., 2001). The cells destined for maintenance culture or for kinetic experiments were cultivated in media without dextran. All experiments were replicated two or three times. The dextrans tested were linear-chain polymers. Dextran of 15 – 20 kDa was from Fluka Biochemika (Buchs, Switzerland) and those of 67 and 160 kDa were from Sigma. Metabolites were determined using an automatic Bioprofile 100 Analyser (Nova Biomedical, Waltham, MA).

2.2.2. Analysis of cell ring

The viability and concentration of free cells in the medium were determined by Trypan Blue [0.2% (w/v)] dye exclusion. Cell rings were sampled by scraping.

Cell ring samples and aggregates were observed by a Steddy-T stereomicroscope (Ceti, Antwerp, Belgium). Viability of cell ring samples was evaluated by fluorescent microscopy using a classic Live/Dead (calcein AM: 2.5 µM, ethidium homodimer: 5 µM) cytotoxicity kit (Molecular Probes, Leiden, The Netherlands).

Percentage of cells in the cell ring and total cell number in the medium were evaluated on the basis of cell protein content according to the Bradford test (Bradford, 1976). The percentage of cells in the cell ring is the number of cells in the cell ring divided by the total cell number (in the medium and in the cell ring).

2.2.3. Infection and assay for seAP activity

Infections were performed in 250-ml shake flasks with 50 ml of YPR medium after total medium changeover. In the first three hours of the infection, cells were left in one-third of the total medium volume with a gentle agitation every 30 min. Both intra- and extracellular enzymatic activities of seAP were determined using protocols from Dee et al. (1997a).

Analyses of virus binding were performed with High-Five cells in YPR medium at a concentration of $5x10^6$ cells/ml and a MOI of 10. Binding was deduced by difference between virus titer in the medium before and three hours after infection. Virus titers were determined by the standard end-point dilution assay in 60-well plates (O'Reilly et al., 1992) with addition of 5-bromo 4-chloro 3-indolyl phosphate to detect the presence of baculovirus expressing seAP (final concentration of 0.11 mg/l).

2.2.4. Determination of cell surface hydrophobicity

The hydrophobicity of a surface is typically determined on the basis of its lack of attraction to water. Therefore, a hydrophobic surface has a smaller attraction to water than, e.g., to a hydrocarbon. The most common methods to evaluate the cell membrane or a bacterial cell wall hydrophobicity is the *bacteria adhesion to hydrocarb*on (BATH) test (Rosenberg et al., 1980). The technique is based on the partitioning of a hydrophobic cell surface at the interface of a biphasic hexadecane-water system after a short vortexing (van den Mei et al., 1991). In our case, we used a modified BATH test developed for insect cells (Wu, 1996): High-Five cells from suspension culture were centrifuged at 800 *g* for 5 min, washed three times with PBS (37 mM Na_2HPO_4, 163 mM NaH_2PO_4, pH 6.2) and resuspended in PBS to obtain a suspension of $1.2 – 1.4x10^6$ cells/ml. One ml of the cell

suspension was added to a polystyrene 12x75 mm tube (Greiner Bio-One, Frickenhausen, Germany) with 1 ml of hexadecane and vortexed for 15 s at room temperature. This results in the formation of droplets of hydrocarbon and if cells are hydrophilic, few or no cells adhere to these droplets. The sample was left over 40 min to reach a stable phase separation. The cell density in the lower aqueous phase was measured with a hemocytometer before and after vortexing. This percentage was used as a measure of cell surface hydrophobicity using the relationship (Wu et al., 1995; Wu, 1996) (Equation 2-1).

$$\text{Cell hydrophobicity } (\%) = 100.\left(1 - \frac{\text{cell density after vortexing}}{\text{cell density before vortexing}}\right) \qquad \text{(Eqation 2-1)}$$

2.2.5. Determination of dextran adsorption on the cell surface

Dextran adsorption was studied by an adaptation of the assays described in Chien et al. (1977) and Wu et al. (1997), previously developed for evaluating, respectively, the adsorption of dextran on human red cells and that of Pluronic F-68 on insect cells. High-Five cells collected from a dextran-free culture were centrifuged at 800 *g* for 5 min, washed three times with PBS and resuspended in PBS. The freshly prepared cell suspension was placed, at a cell concentration of 10^7 cells/ml, in a 1 ml glass tube containing PBS with known concentrations of dextran. The suspensions were then vortexed for 20 s every 10 min. After one hour, the supernatants were centrifuged at 1,000 *g* for 20 min to obtain cell-free supernatants. The dextran adsorbed on cells was determined by difference (concentration of dextran in the supernatant subtracted from the initial concentration). Dextran concentrations in the supernatant fluid were determined, after appropriate dilution, by the anthrone colorimetric assay (Gaudy, 1962; Jermyn, 1975).

2.2.6. Determination of dextran adsorption on silicon and polystyrene surfaces

The adsorption of dextran (67 kDa) on silicon and polystyrene substrata was investigated by XPS (X-ray Photoelectron Spectroscopy). Polystyrene and silicon were used as model substrata for dextran adsorption tests. Polystyrene substrata were prepared by spin-casting polystyrene (PS) (Mw 230,000, Mn 140,000, Sigma; speed 6,000 rpm, acceleration 20,000 rpm.s^{-1}, time 60 s) onto 15 mm diameter circular glass coverslips (VWR: Vel, Leuven, Belgium) from 30 µl of deposition solution (PS 11.5% w/v in toluene, Vel). Prior to polymer deposition, the glass coverslips were cleaned by immersion for one hour in sulfochromic acid (Vel), followed by rinsing (5 X) in milli-Q water (Millipore), and finally ethanol (1 X, Sigma). The discs were finally dried under a nitrogen gas stream.

Silicon substrata were obtained by cutting squares of about 1 cm^2 from silicon wafers (Siltronix, Archamps, France), rinsing with ethanol and drying with a nitrogen flow.

Substrata were kept in contact with a dextran solution [2 ml, 0.1 % (w/v) in milli-Q water] for four hours at 30°C, followed by five consecutive rinsings with milli-Q water and then dried with a nitrogen flow. Control experiments were performed by using milli-Q water instead of dextran solution.

The XPS analysis was performed with a SSI X-Probe (SSX-100/206; Surface Science Instruments, Mountain View, CA) equipped with an aluminium anode and a quartz monochromator, and interfaced with a personal computer running with the S-probe software for data accumulation and treatment (Dufrêne et al., 1999).

2.3. RESULTS

2.3.1. DEXTRAN PREVENTS HIGH-FIVE CELL RING FORMATION

The effects of different concentrations [0.01, 0.1 and 1 % (w/v)] of different dextran types (15-20, 67 and 160 kDa) were studied as to their effects on High-Five cell growth and clumping in batch shake-flask cultures using different media.

Many changes occurred in cell growth and metabolite consumption upon supplementations with dextrans. In the presence of dextran, the maximal cell density of High-Five cells attained was higher than in its absence, with an optimal concentration of 0.1 % (w/v) of 67 kDa dextran. From the three types of dextran tested, the optimal one for growth was that of 67 kDa for all concentrations used, except for 1 % (w/v). For all types of dextran the best concentration was 0.1 % (w/v) (Figure 2-1, Tables 2-1 and 2-3). In Insect-Xpress medium, similar effects of dextran on the decrease of cell ring formation (Figure 2-4) were observed but to a lower extent than in YPR medium, probably because High-Five cells reached lower cell density in Insect-Xpress than in YPR (Table 2-3)

Table 2-1: Effects of type and concentration of dextran on the maximal viable cell density of High-Five cells cultivated in YPR medium in batch. Values indicate the levels of the maximum free cell concentration attained.

maximum reached free cell density (10^6 cells /ml)		dextran concentration (w/v)			
		1 %	0.1 %	0.01 %	control (0 %)
dextran molecular weight (kDa)	15-20	6.3	7.2	6.6	5.0
	67	4.6	**9.6**	7.5	5.0
	160	5.8	6.0	6.5	5.0

With the optimal concentration 0.1 % (w/v) of 67 kDa dextran in YPR medium, the maximal cell density reached was $9.6x10^6$ cells/ml whereas the growth extent in the control without dextran was only $5.0x10^6$ cells/ml (Table 2-1 and Figure 2-1). Moreover, we observed that in the presence of dextran cells seemed to change their metabolism (Table 2-2). In contrast, when Sf-9 cells were used, the supplementation of 67 kDa dextran did not show any change on cell aggregation and had only slight effects on growth behaviour (data not shown). The maximal cell density reached with 0.1 % (w/v) dextran of 67 kDa were $5x10^6$ Sf-9 cells/ml compared to a control without dextran ($4.2x10^6$ cells/ml).

In a further experiment, we cultivated in shake flasks High-Five cells adapted to dextran for 35 successive passages and observed that cells could be cultivated upon long-term passaging in a medium supplemented with dextran (Figure 2-2). After 10 passages, we observed a shrinking and, ultimately, an elimination of the cell ring (data not shown)

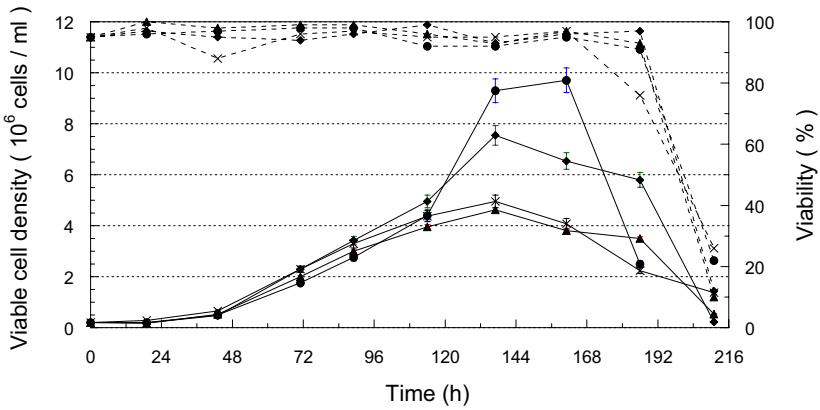

Figure 2-1: Effects of dextran concentration (67 kDa) on growth of High-Five cells in YPR medium. Time course of viable free cell density (full line) and viability (dash line) for the concentration of dextran of 1 % (▲), 0.1 % (●) and 0.01 % (♦) (w/v) compared to an unsupplemented control (X).

Table 2-2: Effects of dextran [0.1 % (w/v), 67 kDa] on substrate consumption and metabolite production of High-Five cells in growth phase in YPR medium. Mean specific rates were estimated based on volumetric rates during growth phase and corresponding viable free cell density.

	YPR medium without dextran	YPR medium with dextran
glucose (10^{-17} mol / cell.s)	- 3.5 ± 0.3 *	- 2.8 ± 0.4
glutamine (10^{-17} mol / cell.s)	- 1.5 ± 0.1	- 0.9 ± 0.1
lactate (10^{-17} mol / cell.s)	0.7 ± 0.1	0.3 ± 0.1
ammonia (10^{-17} mol / cell.s)	1.5 ± 0.2	1.3 ± 0.1

*: experimental error (triplicate experiments)

In T-flasks (data not shown), the percentage of non-adherent High-Five cells was higher (11 %) in the presence of dextran [0.1 % (w/v), 67 kDa] than in its absence (5.5 %). Normally (*i.e.*, in the absence of dextran) the available flask surface is extensively covered by High-Five cells.

After having determined the optimal concentration and type of dextran as 0.1 % (w/v) and 67 kDa, we investigated the attributes of this polymer as an additive in a variety of practical insect cell culture situations: batch bioreactor and infection.

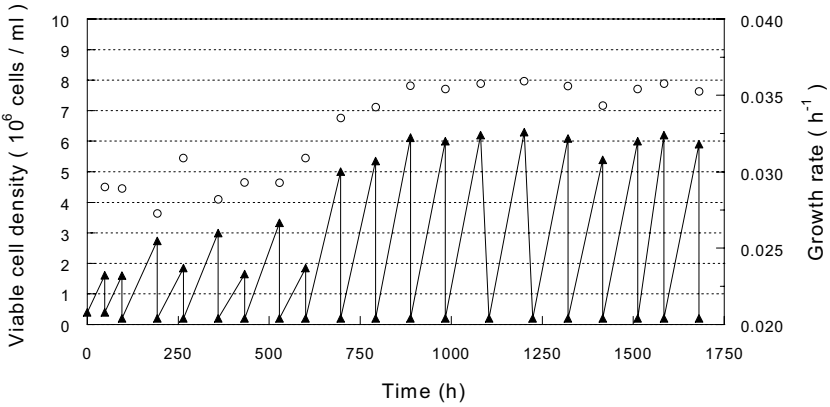

Figure 2-2: Time course of cell density (▲) and growth rate (○) in long-term passaging culture of High-Five of cells to YPR medium supplemented with 0.1% (w/v) dextran (67 kDa) permitting successive subcultures at $6x10^6$ cells/ml.

In a batch bioreactor cultivation, we observed the same type of behavior as in shake flasks, reaching a high density ($7x10^6$ cells/ml) and growth rate ($\mu = 0.025\ h^{-1}$) as well as a notable decrease in the cell ring as compared to the unsupplemented control ($3.9x10^6$ cells/ml), despite the addition of a layer of silicone on the reactor inner surfaces in both cases (data not shown).

In order to confirm in our own system that dextran had no negative effect on infection, a pair of 250 ml shake flasks were infected at $3x10^6$ cells/ml adapted and not adapted to dextran supplemented YPR. In such an infection phase, the presence of dextran had no effect on the yield of r-protein: cultivation with and without dextran, respectively, produced 45 and 42 IU/ml (data not shown). When High-Five cells were adapted to dextran supplemented medium, infections at $8x10^6$ cells/ml could be done (65 IU/ml, Figure 2-3). Cells cultivated without dextran could not be infected at $8x10^6$ cells/ml, because such a cell density could not reached: the maximal cell density attained without dextran ($5x10^6$ cells/ml) produced 52 UI/ml (data not shown).

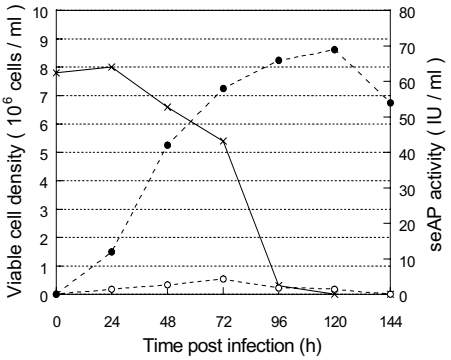

Figure 2-3: Time course of seAP production after infection of High-Five™ cells at MOI of 2 in YPR medium supplemented with 0.1% (w/v) 67 kDa dextran at an initial concentration of $7.6x10^6$ cells/ml: extracellular (●) and intracellular (○) seAP, viable (X) cell.

The binding of virus to the cells in the presence of dextran was not significantly different (99 %) compared to a control (data not shown), whereas sulfated dextran and other sulfated polymers were reported to modify baculovirus infection to varying degrees (Taticek, 1995).

2.3.2. Analysis of cell ring formation

At the same time we observed that the presence of dextran increased significantly free cell density of High-Five, we noticed that this correlated with a decrease in the cell ring (Table 2-3, Figure 2-4).

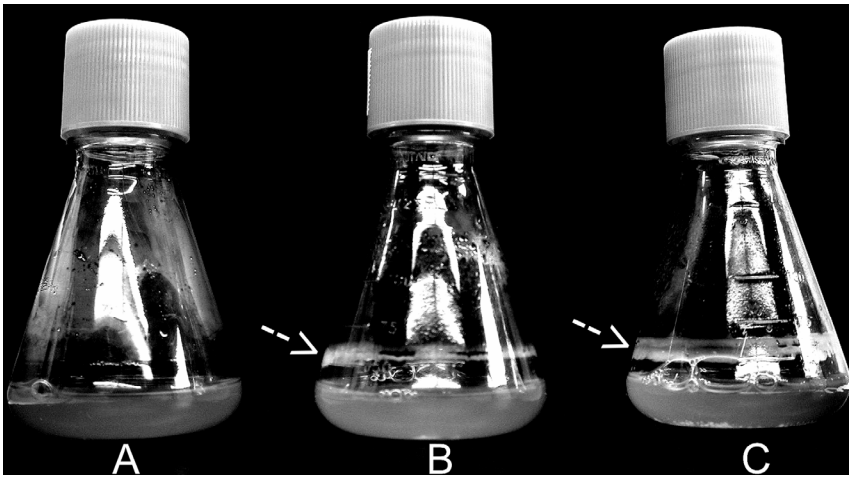

Figure 2-4: Pictures of the cell rings appearing in shake flasks after 168 h of batch culture of High-Five cells cultivated in YPR medium supplemented with dextran [67 kDa, 0.1 % (w/v)] (A), in Insect-Xpress™ medium supplemented with dextran [67 kDa, 0.1 % (w/v)] (B), and in YPR medium without dextran (C). Arrows indicate cell ring.

Table 2-3: Effects of 67 kDa dextran on size of cell ring on walls of shake flasks (125 ml) after 168 h of batch culture.

medium	dextran concentration (w/v)	total cell number in medium * (10^6 cells)	total cell number in cell ring * (10^6 cells)	percentage of cells in cell ring (%)	viability of free cells in medium ** (%)
YPR	1 %	99	2.9	2.8	95
	0.1 %	206	3.9	**1.8**	**97**
	0.01 %	163	14	7.8	93
	0 % (control)	107	24	18.2	94
Insect-Xpress™	1 %	86	7.5	8.0	92
	0.1 %	105	10	8.6	93
	0.01 %	75	20	20.9	95
	0 % (control)	71	38	34.8	91

*: cell number was evaluated on the basis of protein content in cells present
**: viability data were measured at the maximal cell density reached in the batch culture

Consequently, we were interested to understand how this cell ring was formed. We had established by microscopy observations that the cell ring was composed of aggregated cells. However in what state were these High-Five cells? viable, dead, or lysed ? To answer this, we added to a batch culture separately viable, necrotic or lysed cells (Table 2-4). We observe in Table 2-4 that the addition of lysed cells had less significant effects on cell ring increase compared to the addition of necrotic cells.

The addition of lysed cells decreased the viability of free cells more than the addition of necrotic cells. However, the cell ring increased to a lesser extent than when necrotic cells were added. In the case of the addition of necrotic cells, almost all the dead cells added were trapped, after 24 h, in the cell ring. The decrease in viability was due to the increase in the number of cells originating from the artificial (exogenous) addition and from the death of some viable cells. These cells likely died due to the addition of fragments of lysed cells which may cause apoptosis. In this experiment, the use of dextran in the medium had practically no effect on the cell density and viability of suspensions in which necrotic or lysed cells were exogenously added.

In a second experiment (data not shown), $5x10^6$ lysed cells were added to $5x10^6$ viable cells at the beginning of a batch culture (25 ml) in YPR medium devoid of dextran. A cell ring appeared quickly and grew further until the end of culture (at 168 h, 26 % of the cells were in the cell ring). In a parallel cultivation with dextran, the cell ring contained only 16 % of the cells at 168 h.

Table 2-4: Study of cell ring formation. Effects on cell density, viability and percentage of cells in the cell ring, 24 hours after addition of necrotic and/or lysed cells to a batch culture of High-Five cells at a density of $1.0x10^6$ cells/ml (viability 97 %) cultivated in YPR with and without dextran [67 kDa, 0.1 % (w/v)] Ne crotic cells were produced by a rapid increase in temperature (from 27 ℃ to 35 ℃) of a batch culture ($1.0x10^6$ cells/ml, viability 97 %) following by a five-hour cultivation at 35℃. Fragmented lysed cells were obtained by sonication (5 min, 35 Khz) of an identical batch culture.

	cell addition (10^6 cells/ml)		density of viable free cells (10^6 cells/ml)	viability of free cells (%)	percentage of cells in cell ring (%)
	necrotic cells	lysed cells			
YPR medium	none	none	2.1	97	1.3
	1.0	none	1.8	87	27
	none	1.0	1.6	82	11
	0.5	0.5	1.6	81	24
YPR medium with dextran	none	none	2.2	98	0.7
	1.0	none	1.8	85	21
	none	1.0	1.5	77	8
	0.5	0.5	1.7	77	17

2.3.3. PHYSIOLOGY OF INSECT CELLS WITH DEXTRAN

With dextran as medium additive some cell morphology changes appeared. High-Five cells grown in the presence of dextran in YPR medium (average diameter during growth phase: 22.2 µm) were observed to be significantly larger than in its absence (16.5 µm). Assuming that the cells have approximately spherical shape, this increase in cell diameter corresponds to an increase of 125 % in cell volume. Indeed, when protein content was measured, dextran supplemented High-Five cells (1.4 mg protein/10^6 cells) were found to have a 67 % higher cell protein content compared to cells cultured in dextran-free medium (0.8 mg protein/10^6 cells).

Table 2-5: Effects of type and concentration of dextran on cell membrane hydrophobicity of High-Five cells cultivated in YPR medium.

dextran type	dextran concentration (w/v)	cell membrane hydrophobicity (%)
control	0 %	28.9 ± 0.6 *
15-20 kDa	0.1 %	8.1 ± 2.0
67 kDa	1 %	19.9 ± 1.1
	0.1 %	21.6 ± 0.2
	0.01 %	24.4 ± 0.7
160 kDa	0.1 %	32.9 ± 0.6

* experimental error (duplicate experiments)

Cell membrane hydrophobicity decreased for High-Five cells upon increased concentration of 67 kDa dextran in the medium (Table 2-5). For 15-20 kDa dextran at 0.1 % (w/v), the hydrophobicity of cells also decreased, while for dextran of high MW (160 kDa), always at 0.1 % (w/v), the cell hydrophobicity increased compared to a control without dextran (Table 2-5).

2.3.4. ADSORPTION OF DEXTRAN ON CELLS

In order to understand the effects of the dextran polymer on cell membrane hydrophobicity, we measured its adsorption on these cell membranes. The adsorption of the polymer appeared to follow Langmuir's isotherm (Stokes and Evans, 1997) within the concentration range examined (Equation 2-2, Figure 2-5) where θ is the surface coverage, N is the mass (per cell) of dextran adsorbed on cells, N_{max} is the maximum value of dextran adsorbed, C is the concentration of dextran in the solution, and K is the adsorption constant.

$$\theta = \frac{N}{N_{max}} = \frac{K.C}{1 + K.C} \qquad \text{(Eqation 2-2)}$$

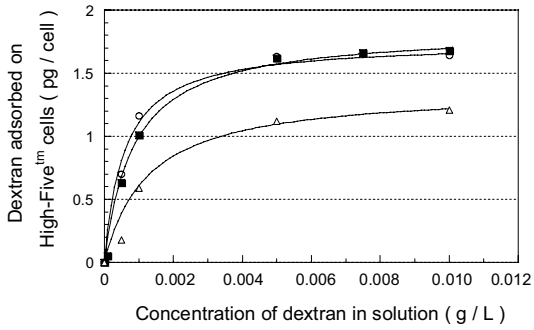

Figure 2-5: Adsorption of dextran on High-Five cells. Lines represent fittings upon determination of N_{max} and K using Langmuir's equation. Experimental data for dextran 15-20 kDa (Δ), 67 kDa (■),160 kDa (○). Initial concentration: 0.02 g/L.

The different N_{max} and K constants were estimated by linear least-squares regression and were found to be 1.37 pg / cell and 80 pg^{-1} / ml for 15-20 kDa dextran, 1.84 pg / cell and 118 pg^{-1} / ml for 67 kDa dextran, and 1.75 pg / cells and 173 pg^{-1} / ml for 160 kDa dextran. The adsorptions of 67 kDa and 160 kDa dextrans on cells seem similar but the adsorption of 15-20 kDa dextran is less intense.

2.3.5. ADSORPTION OF DEXTRAN ON MODEL SUBSTRATA

X-ray Photoelectron Spectroscopy (XPS) was used to assess whether the dextran macromolecules have a tendency to adsorb on the walls of the polycarbonate shake flasks and on the walls of the boro-silicated glass bioreactor. XPS provides a direct chemical analysis of a solid surface, with a monitored depth of about 5 nm. It is therefore well suited to characterize the adsorption of macromolecules on surfaces (Dufrêne et al., 1999).

In order to understand better the potential role of dextran adsorption on bioreactor walls, polystyrene and silicon were used as model surfaces. Silicon (hydrophilic) and polystyrene (hydrophobic) substrata were subjected to dextran adsorption and the conditioned samples were characterized by XPS. As shown in Table 2-6, the surface chemical composition was modified considerably upon dextran adsorption. The decrease of Si atom concentrations on silicon and polystyrene substrata upon dextran adsorption indicates the presence of a layer of dextran, which does not contain Si atoms (Table 2-6). The substantial amount of oxygen present after dextran adsorption on polystyrene can be attributed to the presence of polymer molecules because polystyrene does not contain oxygen. These results indicate that a weak concentration of dextran was adsorbed on the model substrata.

Table 2-6: Surface chemical composition [mole fractions of elements excluding hydrogen, (%)] of silicon and polystyrene substrata prior to and after adsorption of dextran.

Sample	C_{1s}	O_{1s}	Si_{2p}
Silicon (control)	18.04	47.07	38.89
Silicon + 67 kDa dextran *	19.38	47.82	32.81
	19.67	47.12	33.20
PS (control)	98.94	1.06	b.d.l.**
PS + 67 kDa dextran *	93.66	6.34	b.d.l.
	89.39	10.61	b.d.l.

* : data from two independent determinations

** : below detection limits

In presence of dextran, the data were obtained in duplicate.

2.4. DISCUSSION

2.4.1. CELL CULTURE WITH DEXTRAN

In presence of the optimal dextran polymer size (67 kDa) and concentration [0.1 % (w/v)], we observed that High-Five cells had a higher density and viability seemingly due to a decrease in the biomass found in the cell ring of the shake flask walls (Tables 2-1 and 2-3, Figures 2-1 and 2-4). The presence of cells in the cell ring resulted in the decrease of cell numbers in the medium and thus indicates a decrease of the apparent free cell density.

With Sf-9 cells these observations are less crucial, due to the fact that these cells are less prone than High-Five to produce aggregates (Betenbaugh et al., 1991 and Saarinen et al., 1999) and cell ring. At the relatively high agitation rate used (135 rpm), no detectable Sf-9 cell ring was observed in serum-free medium devoid of dextran (data not shown).

In long-term passaging culture in the presence of the above-determined optimal concentration and type of dextran, High-Five cells were able to be subcultured at very high density (Figure 2-2). This is all more remarkable since High-Five is a cell line more sensitive to long-term passaging in suspension culture in serum-free medium than Sf-9 cells (Donaldson and Shuler, 1998).

Under these conditions, High-Five cells were found to produce less lactate (Table 2-2). Cells seemed to have a better use of nutrients and to be in a better state with dextran than without, resulting in a better functioning of the Krebs cycle. Cellular metabolism probably channelled more pyruvate (derived from glucose) to the Krebs cycle than to production of lactate in the presence of dextran (Table 2-2). Nevertheless, these data must be viewed with some caution because the cell metabolism was evaluated on the viable free cells density, and not on the total cell density (free cells + cells in cell ring), which is only measurable at the end of the cultivation.

Concerning production, it was found previously that neutral polysaccharides have no adverse effect on infection or protein production by High-Five cells (Taticek et al., 1997). In our case, dextran seems not to affect the yield of production of recombinant protein (Figure 2-3). The increase of r-protein titre in the presence of dextran is probably only due to an increase of cell density at the time of infection. This polymer does not seem to interfere with virus uptake and thus with the infection.

In conclusion, dextran can be used with High-Five cells both for the growth and production phases, i.e. at all culture stages.

2.4.2. CELL PHYSIOLOGY WITH DEXTRAN

Because the culture environment can modulate cell physiology, it is expected that changes in insect cell culture when dextran is added will probably influence cell physiology (Palomares et al., 2001).

In the presence of dextran High-Five cells appeared to be larger (22 µm, instead of 16 µm). Generally, cell size is determined by metabolic conditions, themselves a function of the nutritional environment, which, in stressful situations, can generate cells smaller than in optimal conditions (Palomares et al., 2001). The change we observed in morphology implies that our cells were in a better state with dextran. That opens the possibility of cultivating cells in better state.

This morphology change appears to be also associated with an alteration in cell membrane hydrophobicity with dextran (Table 2-5). This change in membrane hydrophobicity seems to be a function of the size of the polymer and of its concentration (Wu et al., 1997). The increase in cell membrane hydrophobicity with 160 kDa dextran compared to 67 kDa (Table 2-5) was probably deleterious for the cells and may explain why high MW dextran has previously given poor growth results especially at high concentration (Croughan et al., 1988b).

At the opposite, cells in the cell ring were in a poor physiological state (viability at 32 % for High-Five cells in YPR) most likely due to their compaction and starvation for oxygen and nutrients, and rapidly turned to lysis, as has been also seen with aggregated BHK cells (Moreira et al., 1994). Upon further monitoring, these dead cells proved harmful for the viable free cells in the medium (Tables 2-3 and 2-4), probably by the release of cytoplasmic contents in the medium causing the death of other cells. This was also supported by a significant increase in lactate production, a sign that High-Five cells were stressed (Rhiel et al., 1997; Drugmand et al., 2005).

2.4.3. ADSORPTION OF DEXTRAN ON CELLS AND CULTURE VESSEL SURFACES

Surface active polymers and serum are known to affect strongly the physicochemical properties of insect cell membrane and particularly the cell surface hydrophobicity (Wu et al., 1997). Like many polymers, dextran can be adsorbed on cell membranes (Figure 2-5). All dextrans used in this work showed significant adsorption on insect cells which follows a Langmuir isotherm, implying that the dextran adsorbed on cells forms a layer as a function of its concentration in the surrounding medium (Stokes and Evans, 1997). If the cells are considered as spheres (diameter: 22.2 µm, area: 1.5×10^{-9} m^2),

the amount of adsorbed dextran (1.6 pg / cells for 67 kDa dextran, Figure 2-5) equal to 0.9 mg/m^2, *i.e.* a layer of ~1 nm, suggests that ~ 1 % of the total dextran added into the culture medium (1.0 % w/v) was adsorbed on the cells.

XPS analysis indicated that a weak concentration of dextran was adsorbed on hydrophobic and hydrophilic model substrata. Therefore, if dextran could be adsorbed on such substrata, dextran could also be adsorbed on the culture recipient surfaces: on the inner glass (hydrophilic) and on polycarbonate or polystyrene (hydrophobic) surfaces of bioreactors and shake flasks even if these bioreactor walls are siliconized. Consequently, if dextran was adsorbed on such a solid surface, this would inhibit adsorption of cells by competition for the same surface. In presence of dextran, the decrease in High-Five cell adhesion to T-flask surfaces appears to indicate that the dextran inhibits cell adsorption on recipient walls.

Nevertheless, further characterization is required to assess whether the dextran is adsorbed on cells and recipient walls (monolayer, spaced patches, etc.).

2.4.4. Cell ring formation

Results of the analysis of cell ring formation (Tables 2-3, 2-4 and Figure 2-4) indicate that both dead cells and fragments of lysed cells were implicated in the cell ring formation. In the absence of dextran, some cell fragments coming from the death and lysis of cells (resulting from passive damage) may be adsorbed on the recipient surface at the air-liquid interface (Table 2-4) causing the cell ring formation. This results probably from adsorption of protein and DNA originating from dead cells as proposed by Renner et al. (1993). At the same time, cells, mainly dead (Table 2-4), may be trapped on this cell ring under formation (Table 2-4) due to the centrifugal force caused by the rotary agitation of the shake flasks and bioreactors. This increases the mass of the cell ring. These cells form a compact mass with a necrotic core causing further cell lysis and release of cytoplasmic contents in the culture medium leading-to death of some cells in the medium, perhaps by apoptosis.

The cause-to-effect relationship between cell ring and apoptosis is unknown, yet it has been suggested that the dispersion of cell clumps can diminish nutrient limitation and waste accumulation in cell clumps, which normally trigger apoptosis in CHO culture (Zanghi et al., 2000).

In contrast, in the presence of dextran, this cell ring decreased. We can hypothesize the following two mechanisms suggested by our data to explain the indirect role of dextran on High-Five cell density and viability. First, the presence of dextran decreases cell ring formation by its simultaneous adsorption on both cells and recipient surfaces. The weak

proportion of polymer adsorbed on cells and vessel walls indicates that practically all of the dextran added is free in the medium. Hence, the decrease in cell ring formation may be due to competition between dextran free in the medium and dextran adsorbed on cells and surfaces (Stokes and Evans, 1997). The second hypothetical mechanism is that the dextran inhibits the adsorption of DNA and/or protein on cells and/or culture surfaces, preventing extensive cell ring formation. This mechanism can also explain the formation of only a few aggregates during cultivation with dextran-supplemented medium. These mechanisms may explain the increase in non-adherent cells in cultivations with dextran within T-flasks that was found in this study.

Although dextran at the concentration of 0.1 % (w/v) would not affect medium viscosity (Gerson and Kole, 2001) and medium osmolarity (data not shown), shear stress-protective effects of dextran on insect cells may also contribute to the diminishing of the cell ring (Goldblum et al., 1990), even though at the optimal concentration found in this study [0.1 % (w/v)] dextran would be expected to have no significant effect on alleviating shear-related damage on hybridoma (van der Pol and Tramper, 1998).

We suggest that the favourable influence of this dextran [67 kDa, 0.1 % (w/v)] on High-Five cell as a deterrent to ring formation compared to other types of dextran is due to a more extensive adsorption of this polymer on the cell membrane (Figure 2-5), to the modest change of cell membrane hydrophobicity (Table 2.5) with dextran of intermediate MW and to a lower toxicity, which is known to increase with the MW (Aunins, 1989). The concentration optimum probably results from a compromise between efficiency (Tables 2-1 and 2-3) and toxicity, which increases with concentration.

2.5. CONCLUSION

In sum, the decrease of the cell ring may be a consequence of cumulative and indirect effects of dextran as medium additive. Dextran inhibits this cascade of cell ring formation probably thanks to its adsorption capacities on cells (Figure 2-5) and culture surfaces (Table 2-6). By decreasing the cell ring formation on culture flasks, optimal dextran type and concentration [0.1 % (w/v) and a MW of 67 kDa] contributes positively on the physiological state and viability of High-Five cells. The presence of dextran indirectly contributes to an increase of the apparent density of free High-Five (Figure 2-1 and Tables 2-1 and 2-3), by diminishing the percentage of cells present in the ring. The increase in viability results in all probability from the decrease of the deleterious compact mass and necrotic core that typically forms in the cell ring.

Dextran is a neutral polymer, inexpensive, very effective in decreasing cell ring formation, and non-inhibitory of viral infection. Therefore, we recommended the use of dextran for insect High-Five insect cell culture and we suggest the testing of dextran in the cultivation of other animal cells which may also suffer from cell ring in shake flasks culture.

Acknowledgments:

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3 PART II: INSECT CELL PHYSIOLOGY AND METABOLISM

Chapter 3 : Comparison of methods and identification of environmental factors causing insect cell death

3.1. INTRODUCTION

In suspension cultivation, especially in bioreactors, cell death is a critical factor limiting the productivity of cultured animal cells. The characterization of the death process and the identification of factors affecting it, may lead to new approaches to optimize cell-based productions (Singh et al. 1994). There are two ways by which cells can die: either necrosis or apoptosis

Necrosis is a passive way which is characterised by the modification of the non-specific permeability of the cytoplasmic membrane, the swelling of cellular organelles, the release of lysosomal content into the cytoplasm causing the disorganization of nuclei, the fragmentation of the plasma membrane and the release of the cytosol contents into the supernatant (Al-Rubeai 1998; McKenna et al. 1998).

Apoptosis is an orderly cellular self-destruction mechanism that is activated in response to various signals. It is an organized process, characterized by the loss of the mitochondrial transmembrane potential and the release of cytochrome c out of the mitochondria, by the condensation of chromatin, the degradation of intracellular DNA into oligonucleosome-sized fragments (180-200bp), the flipping of phosphatidelserine in the plasma membrane, the latter's degradation and cell blebbing, the production of apoptotic bodies and premature cytolysis (Al-Rubeai 1998; Lacount and Friesen 1997; McKenna et al. 1998).

In higher eukaryotic (animal) cells, two ways are known to activate the apoptosis: the "receptor way" and the "mitochondrial way" (Figure 3-1). The receptor way involves links between signals (ligands) and surface receptors (e.g. TNF-R: Tumor Necrosis Factor Receptor). The receptors activate a complex cascade of production of upstream activator-type caspases until the activation of pro-caspase-8 (DREDD for *Drosophila* cells) that is involved in the production of pro-caspase-3 (DrICE for *Drosophila* cells), the first downstream effector-type caspase (Al-Rubeai 1998; Clarke and Clem 2003). This caspase activates other effector caspases which are implicated into the morphological modifications of the cells (DNA fragmentation, externalisation of phosphatidylserine).

The mitochondrial way requires the release of cytochome c out of the mitochondria. After that, cytochrome c binds to the APAF-1 (DARK in *Drosophila* cells) and the activator-type pro-caspase-9 (DRONC in *Drosophila* cells) to produce the apoptosome complex, which is involved in the activation of pro-caspase-3 (DrICE) (Al-Rubeai 1998; Clarke and Clem 2003). Thus, the activation of caspase-3 could be brought about by either the receptor way or the mitochondrial way. Moreover, caspase-8 is able to cleave the

pro-apoptotic BIB protein allowing the release of cytochrome c. The pro-caspase-3 (DrICE) can be activated by the two ways. Apoptosis is a complex balance between pro-apoptotic and anti-apoptotic proteins. Among them, CED-9 inhibits the APAF-1 and BCL-2 inhibits the release of the cytochrome c.

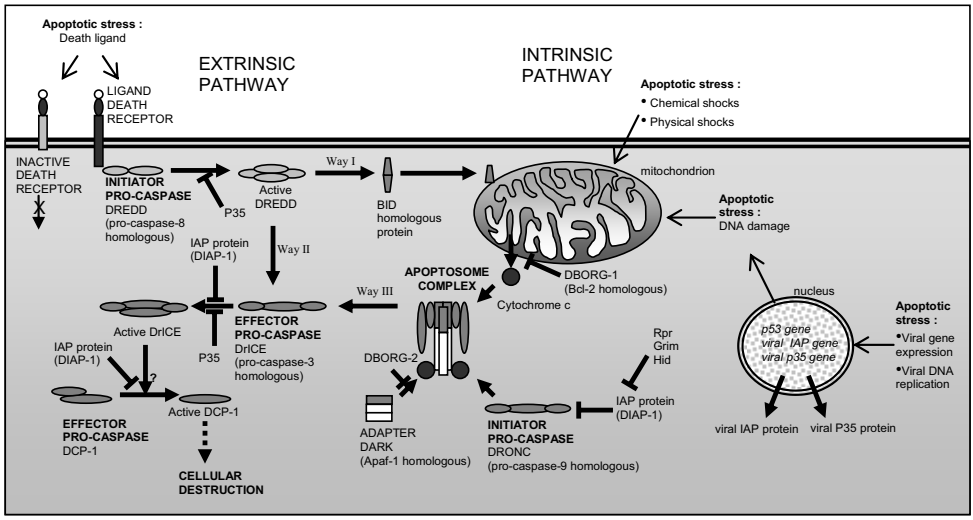

Figure 3-1: Apoptosis pathway in insect cells

Unfortunately, the molecular mechanisms of apoptosis in invertebrate cells remain partially unknown (Figure 3-1). Today, seven caspases analogous to mammalian activator and effector-type caspases are identified in *Drosophila* (vs. 14 for mammalian cells), one Sf-Caspase-1 (analog to caspase-3) and the *Drosophilia* DrICE and DCP-1 have been detected in *Spodoptera frugiperda* (Ahmad et al. 1997; Clarke and Clem 2003). Various apoptotic receptors have been detected in *Drosophila* cells. A gene analogous to the human *p53* gene involved in the repair of damaged DNA has been detected in *Drosophila*. Analogs of APAF-1, BCL-2 and CED-9 gene products have been detected in *Drosophila* cells, and respectively named DARK/HAC/DAPAF-1, BEBCL/DBORG-1/DROB-1 and BUFFY/DBORG-2 (Richardson and Kumar 2002; Tittel and Steller 2000).

The baculoviruses specific for invertebrates are known to inhibit apoptosis in insect cells. Expression of the *p35* or *iap* (Inhibitor of Apoptosis Proteins) genes, two unrelated baculovirus apoptotic precursors, is known to block virus-induced apoptosis and consequently to restore virus replication and production of viral progeny (Lacount and Friesen 1997). Invertebrate (*Drosophila*) cells have *rpr*, *grim* and *hid* (Head Involution Defect) genes whose proteins could inhibit the activity of the antiapoptotic IAP proteins and

activate apoptosis via the "receptor way". Apoptosis in insect cells has been reviewed by Hengartner (1996), Clem (2001), Clarke and Clem (2003) and compared with other animal cells by Tittel and Steller (2000).

Several authors separate apoptotic cells based on cell morphology into two populations: the early and the late apoptotic cells (Cowger et al. 1999; Mercille and Massie 1994). The early apoptotic cells have their chromatin condensed but their membranes intact, whereas, on the contrary, late apoptotic cells have already damaged membranes. On the other hand, necrotic cells have their membrane damaged without chromatin condensation. Although apoptosis is a reversible event, when morphological modifications are visible, the phenomenon becomes irreversible. During cultivation, cells with these different aspects may be present together.

The aim of the present work was to investigate methods for detection of cell death in insect cells cultured in suspension and to study the effect of environmental factors at its root. No extreme assaults but only moderate chemical, physical and metabolic stresses that could occur under typical cultivation conditions were investigated, including temperature, pH, agitation rate, presence/absence of Pluronic F-68 in the medium, and concentrations of glucose, glutamine (Gln), oxygen, ammonia and lactate.

3.2. MATERIALS AND METHODS

3.2.1. CELL CULTURE AND APOPTOSIS INDUCTION

High-Five™ and Sf-9 insect cell lines were used. Sf-9 cells were bought from Invitrogen (Carlsbad, CA), whereas High-Five cells were a gift from the Laboratory of Virology (University of Wageningen, The Netherlands). A recombinant *Autographa californica* nuclear polyhedrosis virus (AcMNPV) baculovirus encoding β-galactosidase (β-gal) was used (Invitrogen).

Cells were maintained in exponential phase in 125-ml shake flasks (Corning, Acton, MA) with 25 ml of medium inoculated with $0.2x10^6$ High-Five cells/ml or with $0.3x10^6$ Sf-9 cells/ml. The cell growth was examined, in triplicate, in the above shake flasks or in 175 cm^2 T-flasks at 28°C and 130 rpm (unless specified otherwise), using YPR medium (Ikonomou et al. 2001) supplemented with 0.1 % (w/v) of 67 kDa dextran. For bioreactor studies, a Celligen-Plus™ bioreactor (New Brunswick Scientific, Edison, NJ) of 1 L working volume agitated at 65 rpm was used. Inoculation of either cell type was done at $0.2x10^6$ cells/ml. Temperature was set up at 28°C, the dissolved oxygen (DO) at 50 % of air saturation, and the pH at 6.2.

Concentrations of lactic acid, ammonia and pH were determined using a Bioprofile 100 Analyser (Nova Biomedical, Waltham, MA). Glucose was determined by a glucose oxidase-based enzymatic kit (Boehringer, Mannheim, Germany).

During growth, the cell density and viability were estimated by Trypan Blue dye exclusion (0.2% w/v final concentration). For positive control experiments, apoptosis was induced using 30 mM sodium butyrate or 5 mM hydroxylamine.

3.2.2. ANTIOXIDANT CAPACITY

The antioxidant capacity of the medium was investigated using an ORAC (oxygen radical absorbance capacity) test according to the protocols of Huang et al. (2002) and Ou et al. (2001) using an Ascent F.L. Fluoroscan spectrofluorometer (Labsystems, Vantaa, Finland) with the Ascent 2.4.2 software. Trolox (Sigma) was used as standard and antioxidant capacity was expressed by comparison to Trolox.

3.2.3. LACTATE DEHYDROGENASE ACTIVITY

The lactate dehydrogenase (LDH) activity released by the cells in the supernatant was determined using the Cytotoxicity Detection Kit LDH of Roche (Mannheim, Germany). A pure L-lactate dehydrogenase from rabbit muscle (Sigma) was used for calibration and standardization. The LDH units were converted into the amount of lysed cells after interpolation from a calibration curve. One unit of LDH corresponds to ca. $0.33x10^6$ High-Five and $1.57x10^6$ Sf-9 cells lysed, respectively.

3.2.4. STAINING WITH FLUORESCENT DYES

Cell analysis was performed using fluorescent dye microscopy. Samples of suspended cells were collected, counted, diluted to obtain $0.5x10^6$ cells/ml and stained. Coloured cells were counted using a hemotocytometer to estimate the percentages of each cell type. A classic Live/Dead cytotoxicity kit (Molecular Probes, Leiden, The Netherlands) with calcein AM (2.5 µM) and ethidium homodimer (Eth) (5 µM), 30 min of incubation, was used to distinguish the viable (coloured in green) from the dead cells (red). Staining with acridine orange (AO) (200 µg/ml) and propidium iodide (PI) (15 µg/ml) (Sigma) during 15 min of incubation were used together to investigate the apoptosis (respective fluorescence green and red). According to Mercille and Massie (1994), cells were classified into four species: viable, early- and late apoptotic, and necrotic based on aspect of the cytoplasm and permeability to PI. Rhodamine 123 (Rh123) (Sigma) was used, at 2 µg/ml with 15 min of incubation, to distinguish the apoptotic cells (diffuse staining) from viable cells which have green stained mitochondria. Annexin-V-FITC was used at 0.5 µg/ml to estimate the numbers of apoptotic cells (stained in green). The Rh123 or

Annexin-V-FITC were used, combined with PI, to distinguish the viable cells from apoptotic and necrotic cells. Confocal microscopy was employed to investigate the population of cells stained with AO/PI dyes. It allowed to increase the resolution and to separate the respective emission fluorescence of AO and PI using 540 and 580 nm filters. The system consisted of an emission source (Bio-Rad MRC 1024, Bio-Rad, Hemel, Hempstead, UK) with an excitation filter at 488 nm, a microscope (Zeiss Axiovert 135 M, Carl Zeiss AG, Göttingen, Germany).

3.2.5. DNA FRAGMENTATION

The fragmentation of DNA was explored using a DNA ladder protocol according to Verhaegen et al. (1992) and Baust et al. (2000). Samples of suspended cells were collected, counted, centrifuged to obtain $3x10^6$ cells/ml and lysed. Subsequently, DNA was extracted, added to bromophenol blue, and loaded on a 1 % (w/v) agarose gel with 1 µg/ml of ethidium bromide. Migrations of DNA were followed for one h at 200 V. DNA molecular weight markers XIV (Roche) were used. The intensity of bands on the gel was analyzed with "NIH image", a software developed by the Research Service Branch of the National Institute of Health (Bethesda, MD).

For detection of DNA fragmentation by ELISA (Roche), cells in growth phase were incubated at $2\text{-}4.10^5$ cells/ml with 10 µM of BrdU (Sigma) for 18 h at 28°C. During this incubation, the BrdU was incorporated into the DNA. Afterwards, the supernatant was centrifuged (250xg, 10 min) and the cells were placed into fresh medium. Samples were collected during cell growth, and the fragmentation of the labelled DNA was analysed by ELISA according to the Roche protocol.

3.2.6. DETECTION OF CASPASES

Detection of caspase-3 produced during the apoptosis of the cells was based on an ApoAlert™ caspase colorimetric assay (BD Biosciences Clonetech, Palo Alto, CA). In accordance with the manufacturer's instructions, 1.5 ml of $2x10^6$ cells/ml was collected and centrifuged (400xg, 10 min), the pellets were frozen at –80°C before detection, and finally the caspase-3 activity was assayed.

3.3. RESULTS

3.3.1. Comparison of Methods

The death of High-Five and Sf-9 cells was investigated using the calcein AM/Eth, AO/PI, Rh123/PI, Annexin-V-FITC/PI fluorescent dyes, Trypan Blue dye exclusion, assay of LDH release, ELISA DNA fragmentation, DNA ladder, and caspase-3 activity detection.

With the AO/PI assay, the distinction between cell types could be made depending on the state of the nucleus and the permeability of the plasma membrane (Figure 3-2). According to Mercille and Massie (1994), viable cells have an intact membrane, while early and late apoptotic cells have their chromatin condensed, and, finally, necrotic cells have diffuse chromatin and damaged membrane. The distinction between early and late apoptotic cells is made on the permeability of the membrane, which is high for late apoptotic cells while early apoptotic cells remain impermeable to PI.

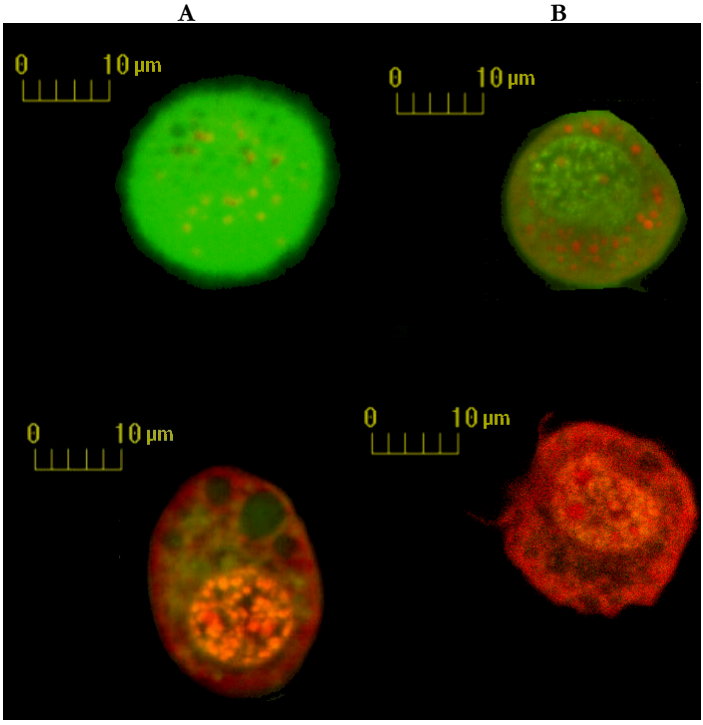

Figure 3-2: Confocal microscopy pictures of High-Five cells stained with AO/PI. A: viable cells, diffuse chromatin stained with AO, intact membrane and cytoplasm stained with AO; B: early apoptotic cells, condensed chromatin stained with AO, damaged membrane and cytoplasm staining with AO and PI; C: late apoptotic cells, condensed chromatin stained with PI, damaged membrane, cytoplasm stained with low AO and high PI; D: necrotic cells, diffuse chromatin stained with PI, high damaged membrane, cytoplasm stained with PI.
Superposition with Confocal Assistant of pictures obtained using 540 (red) and 580 nm (green) emission filters.

The Rh123 and Annexin-V-FITC were used in combination with PI to distinguish the viable cells (mitochondria stained with Rh123, no staining with Annexin-V-FITC) from apoptotic cells (staining of cytoplasm with Rh123 and Annexin-V-FITC), and from necrotic cells (staining with both PI and Rh123 and Annexin-V-FITC). The cytotoxicity of these dyes was estimated by addition of the concentration specified in Materials and Methods, for one hour, to High-Five and Sf-9 cells at 3-4x10^6 cells/ml and 95 % viability (Trypan Blue). One hour later, the viability measured was 80 % with AO, 80 % with PI, 85 % with Annexin-V-FITC, and 65 % with Trypan Blue. The detection limit of these microscopic observations was estimated at 1x10^4 cells/ml for each cell type, whereas the relative error on the percentage of a cell type using the AO/PI, Rh123/PI or Annexin-V-FITC/PI was estimated at 1.5% (statistical analysis on multiple measurements).

Neither confocal nor fluorescent microscopy gave any indication of apoptotic bodies with High-Five cells. Concerning Sf-9 cells, similar microscopic observations were made (data not shown) except for the detection of apoptotic bodies in addition to blebbing cells.

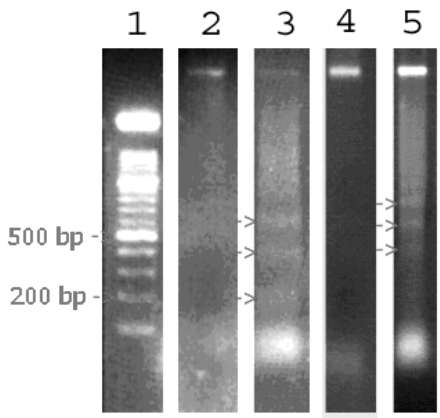

Figure 3-3: DNA ladder of High-Five cells in growth phase upon cultivation in YPR medium (55 mM glucose, 12 mM Gln) (lane 2), in modified YPR medium containing only 12 mM glucose and 4 mM Gln (lane 3), in modified YPR medium containing only 4 mM Gln (lane 4) or in modified YPR medium containing only 12 mM glucose (lane 5); molecular weight markers (lane 1). Arrows indicate 200 bp, 500 bp and rungs of the DNA ladder.

Concerning the DNA ladder, the detection of DNA fragments of ca. 180-200 bp, 350-400 and 500-600 bp indicated that each rung of the ladder was constituted of oligonucleosome-sized fragment multiples of 180-200 bp (Figure 3-3). The detection limit of the DNA fragmentation was estimated at 0.4x10^6 +/- 0.15 apoptotic cells/ml for both cell lines (data not shown).

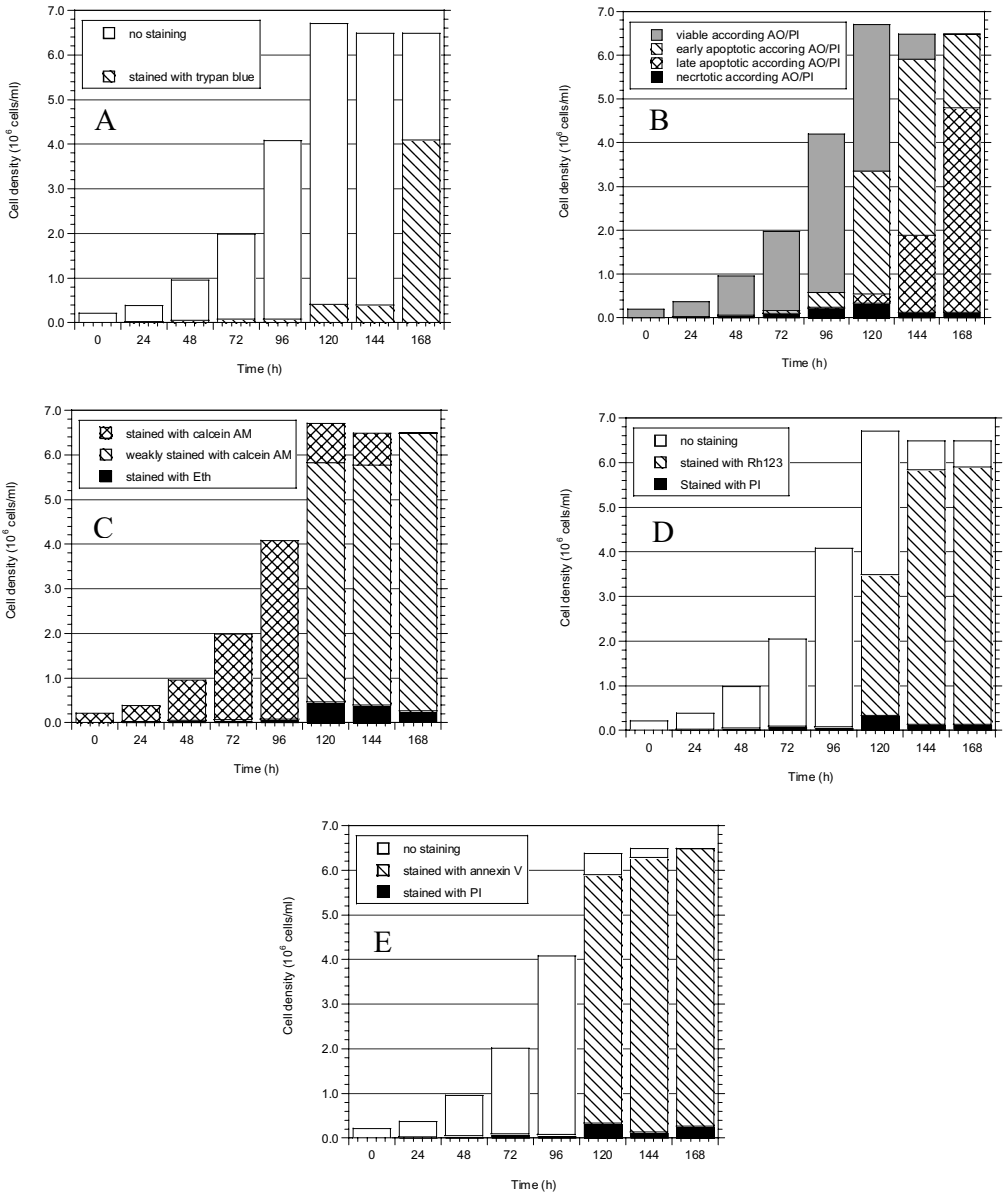

Figure 3-4: Evolution of cell populations during a batch cultivation of High-Five cells in shake-flasks (data represent the mean of triplicates). Cells were stained using Trypan Blue dye exclusion (panel A), AO/PI assay (populations were classified according Materials and Methods) (panel B), calcein AM and ethidium (panel C), Rh123 and PI (panel D) and annexin-V-FITC and PI (panel E).

The quantitative estimation of the numbers of apoptotic cells using the analysis of band intensity of the DNA ladder electrophoresis gel gave an error of between 50 and 80 % (by comparison to AO/PI test). The detection limit of the ELISA fragmentation test was estimated at $0.2x10^6$ (High-Five) and $0.8x10^6$ (Sf-9) apoptotic cells per ml (data not shown).

A comparison of all of these methods on a High-Five cell batch cultivation in shake flasks (triplicate) is given in Figure 3-4. All of the fluorescent dyes detected the activation of the apoptosis between 96 and 120 h of cultivation, but not the Trypan Blue dye exclusion.

Among these fluorometric assays, the AO/PI assay was able to detect apoptosis in insect cell culture earlier than the others. But, during the stationary phase (at 120 h), it seemed to underestimate the number of early apoptotic cells compared to Annexin-V-FITC and Rh123 (Figure 3-4). The AO/PI was the only test able to distinguish between the early and late apoptotic High-Five cells (Figure 3-4). At the end of the cultivation (168 h), the percentage of necrosis detected by PI and Eth (between 4 and 9 %) was lower than the percentage of cells stained by Trypan Blue (Figure 3-4).The distinction between viable and apoptotic Sf-9 and High-Five cells with the live/dead test (calcein AM/Eth) was subjective since it was based on a visual discrimination between highly and weakly stained cells (data not shown).

During the cultivation, the activity of the caspase-3 slowly increased from 0 to 4.7 caspase units/10^6 cells, at 120 h, and reached 7.6, 27.4 and 26.6 caspase unit/10^6 cells, respectively at 144 h, 168 h and 192 h (data not shown). During the cultivation, samples for DNA ladder assay were withdrawn daily. A smear of DNA fragmentation had been detected from 96 h, while rungs of the ladder were visible from 144 h (Figure 3-5).

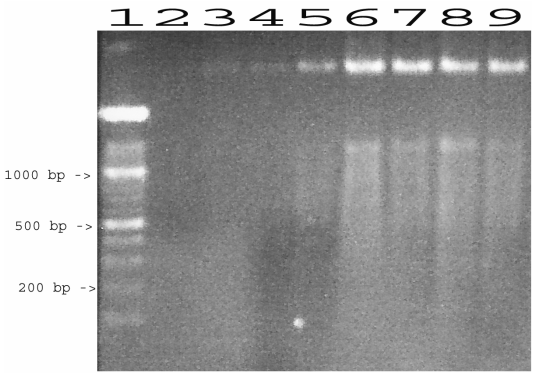

Figure 3-5: DNA ladder pattern of a batch of High-Five cells in growth phase; lanes 2 to 5 are respectively the ladders after 24, 48, 72 and 96 h of cultivation, lanes 6 to 9 are the ladders of cells diluted by 2 at 120 h , 144 h, 168 h and 192 h, whereas lane 1 represents molecular weight markers.

In another experiment, the cell density/viability of a High-Five batch cultivation was investigated using Trypan Blue, while the cell types were followed using AO/PI and LDH assays (Figure 3-6). Viable cells reached a maximum at 96 h and remained stable for one day. Upon reaching the stationary phase, cells began to die by apoptosis. The appearance of late apoptotic cells (168 h and 192 h) was coupled with the drop in the number of early apoptotic cells and no late apoptosis was detected before the detection of early apoptosis. The detection of dead cells by LDH assay began at 144 h, and increased between 168 h and 192 h, reaching by the end of cultivation a density equal to the maximal cell density (120 h). This indicated that the total cell density was stable during the stationary phase. During the death phase, few necrotic cells were observed (below $0.3x10^6$cells/ml throughout the cultivation), but, rather, cells entered massively into an apoptotic state.

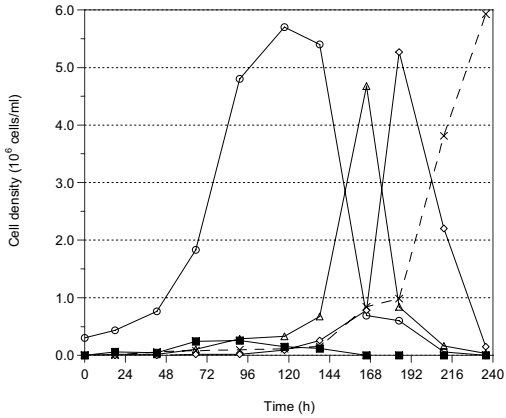

Figure 3-6: Evolution of cell population during a batch cultivation of High-Five cells in a Celligen-Plus bioreactor (mean of duplicates). Cell types were classified using the AO/PI test and the LDH release for lysed cells. Time course of cell populations: viable cells (○), early apoptotic cells (Δ), late apoptotic cells (◊), necrotic cells (■) and lysed cells (X).

The apoptotic behavior of High-Five and of Sf-9 cells cultivated under adherent conditions in T-flasks was also investigated (data not shown). Seventy-two hours after inoculation, detached cells were not abundant (~$0.4x10^6$ cells/ml for both cell lines), but nearly all of these detached cells were late apoptotic (~70 % for both cell lines) and necrotic (12 % for High-Five cells and 7 % for Sf-9 cells). Adherent cells were viable (89 and 84 %, respectively for High-Five and Sf-9 cells) and only very few of them seemed to be early apoptotic.

3.3.2. FACTORS AFFECTING CELL DEATH

Various environmental factors including temperature, pH, agitation rate, presence/absence of Pluronic F-68 in the medium, and concentrations of glucose, Gln, ammonia and lactate were tested as to their effects on insect cell death.

The influence of temperature on Sf-9 and High-Five cell death was investigated using the AO/PI test (Table 3-1). Cells were inoculated in fresh medium and cultured in batch culture between 25°C and 35 °C. As shown in Table 3-1, one day after the inoculation at 32°C and 35°C, a decrease of viability and a high increase of numbers of necrotic cells was observed in both cell lines (data not shown). . High-Five cells grew more rapidly than Sf-9 cells upon a temperature increase. The growth phase of both cell lines decreased when cells were cultivated at 25°C, but without significant decrease of viability. At all temperatures and for both cell lines, few necrotic cells were observable during the death phase. In another experiment, upon long term passaging (10 passages) at 32°C (data not shown), a slow decrease of the duration of the growth phase was observed for both cell lines but there was no change in the percentages of necrotic and apoptotic cells in growth phase.

Table 3-1: Effect of temperature stress on insect cell apoptosis. Cells were inoculated in fresh YPR medium and cultivated during the whole period of batch cultivation at the indicated temperature. Percentages of viable and (early plus late) apoptotic cells were obtained using the AO/PI test.

Temperature (°C)	Maximum cell density (10^6 cell/ml)	μ (h^{-1})	Necrotic cells at the end of the growth phase (%)	Apoptotic cells at the end of the growth phase (%)
High-Five				
25	5.2	0.018	3	7
28	5.8	0.027	2	9
30	5.9	0.033	1	12
32	6.3	0.035	0	14
35	4.5	0.040	2	27
Sf-9				
25	7.5	0.022	3	8
28	10.5	0.028	2	7
30	9.9	0.026	4	10
32	9.8	0.030	3	7
35	7.2	0.031	5	12

The effects of the presence/absence of Pluronic F-68 in the medium and of the agitation rate on the death of High-Five cells were investigated using the AO/PI test (Table 3-2). When the concentration of Pluronic diminished while keeping the agitation rate constant at 130 rpm, the maximal cell density decreased. Without Pluronic, 24 h after

the inoculation, all the cells were necrotic (data not shown). When the agitation rate increased, the cell viability decreased accompanied by a higher increase of necrotic than apoptotic cells. At the higher agitation rates of 170 and 200 rpm, the presence of cell aggregates increased in the culture (data not shown). The increase of agitation did not increase the number of apoptotic cells. Similar results were also observed with Sf-9 cells (data not shown).

Table 3-2: Effect of agitation and Pluronic F-68 concentration on the High-Five cell apoptosis. Cells were inoculated at $1.7x10^6$ cells/ml and cultivated during the whole period of the batch cultivation under the indicated culture conditions. Percentages of viable and (early plus late) apoptotic cells were obtained using the AO/PI test.

Culture conditions		Maximum cell density (10^6 cell/ml)	Necrotic cells at the end of the growth phase (%)	Apoptotic cells at the end of the growth phase (%)
Pluronic (g/l)	Agitation (rpm)			
0	130	1.8	98	2
0.01	130	3.0	4	15
0.25	130	4.2	2	19
0.1	130	6.0	1	12
0.1	150	7.5	7	9
0.1	170	6.2	8	10
0.1	200	4.5	8	14

The influence of medium additives on High-Five cell death was also investigated (Table 3-3) using extract (peptone)-free medium, YPR medium and serum-containing medium. Without the extracts Primatone and Yeastolate, the High-Five cells reached a density lower than the one obtained with complete YPR medium, with a higher percentage of apoptotic cells. One day after the switch from YPR to extract-free media, High-Five cell growth stopped, cells died rapidly and a cell ring appeared around the shake flask walls (data not shown). As expected, the presence of serum and hydrolysates in the medium influenced positively cell growth and viability.

The presence of peptones (Primatone and Yeastolate) in the medium seemed to have equal anti-apoptotic effect as serum. Yeastolate seemed to have a higher growth promoting effect than Primatone, which, in turn, seemed to have a slightly higher antiapoptotic effect than Yeastolate. Similar results were obtained with Sf-9 cells (data not shown).

The antioxidant capacity of the medium components was also investigated (Table 3-4) to understand the beneficial effect of Primatone and Yeastolate on cell growth. According to the results of the ORAC test, the Yeastolate (at 6 g/l), Primatone (at 5 g/l) and

IPL-41 were responsible for 45, 20 and 33 % of the antioxidant capacity of YPR medium, respectively.

Table 3-3: Effect of Yeastolate and Primatone on High-Five cell apoptosis. Cells were inoculated at $1.7x10^6$ cells/ml in the indicated media and cultivated in batch culture. Percentages of viable and (early plus late) apoptotic cells were obtained using the AO/PI test.

Medium	Maximum cell density (10^6 cell/ml)	Viable cells at the end of the growth phase (%)	Apoptotic cells at the end of the growth phase (%)
Extracts- free medium	1.3	71	18
Yeastolate-free medium	5.4	87	11
Primatone-free medium	3.8	90	7
Extracts-free medium with 5% serum	2.5	82	9
YPR medium with 5% serum	6.6	89	11
YPR	6.2	88	8

Table 3-4: Antioxidant capacity of YPR medium.

Compounds	Antioxidant capacity (µmol Trolox equivalent /l)
IPL-41 medium	4300
Glucose (7.5 g/l)	0
Glutamine (0.5 g/l)	0
Pluronic (1g/l)	0
Lipids (2.5 g/l)	0
Dextran (1g/l)	5
Yeastolate (6 g/l)	5905
Primatone (5 g/l)	2580
Extracts- free YPR	4305
YPR medium	12980

The effect of by-products on the insect cell death was investigated using cultures with exogenous addition of ammonia (ammonium chloride, Sigma) and lactate (sodium L-lactate, Sigma) into the fresh medium (pH was adjusted at 6.2) before inoculation (Table 3-5) designed to obtain by-product concentrations similar to those found at the end of a standard batch culture. The higher the ammonia concentration in the medium, the higher the percentage of apoptotic High-Five cells that was found at the end of the growth phase. The presence of lactate seemed to shorten the growth phase and the maximal High-Five cell density reached, whereas ammonia did not. With Sf-9 cells, ammonia decreased the growth rate (data not shown). The influence of the pH appeared to be higher on the

decrease of the maximal cell density and growth phase duration than on the apoptosis of the High-Five cells (Table 3-5, confirmed by a DNA ladder).

Table 3-5: Effect of ammonia, lactate and pH on High-Five cell apoptosis. Cells were inoculated at 1.7x10^6 cells/ml in YPR medium to which exogenous ammonia and lactate were added or initial pH was changed. Percentages of viable and (early plus late) apoptotic cells were obtained using the AO/PI test.

Initials conditions			Maximum cell density (10^6 cell/ml)	μ (h^{-1})	Necrotic cells at the end of the growth phase (%)	Apoptotic cells at the end of the growth phase (%)
Ammonia (mM)	Lactate (mM)	pH				
0	0	5.8	4.7	0.021	3	5
0	0	6.0	5.3	0.0235	2	4
0	**0**	**6.2**	**5.4**	**0.024**	**2**	**2**
0	0	6.5	5.9	0.026	2	5
0	0	6.8	4.9	0.022	3	6
20	0	6.2	5.4	0.024	2	10
0	12.5	6.2	4.8	0.021	3	6
20	12.5	6.2	4.9	0.022	2	29

Table 3-6: Effect of glucose, Gln and oxygenation on High-Five cell apoptosis. Cultivation of cells in low glucose (12 mM) medium, low Gln (4mM) medium, low glucose (12 mM) and low Gln (4mM) medium, hypoxia condition (5 % DO) and hyper-oxygenation condition (400 % DO) were compared to the standard condition (YPR medium, 50 % DO, glucose 55 mM, Gln 12 mM) in a Celligen-Plus bioreactor. Percentages of viable and (early plus late) apoptotic cells were obtained using the AO/PI test.

	Conditions	Maximum cell density (10^6 cell/ml)	Necrotic cells at the end of the growth phase (%)	Apoptotic cells at the end of the growth phase (%)
Sf-9 cells	**Standard conditions: YPR** (50 % DO)	**10**	**2**	**6**
	Low glucose medium (50 % DO)	7.8	5	78
	Low Gln medium (50 % DO)	8.2	3	8
	Low Glucose and low Gln medium (50 % DO)	6.9	13	87
	Hypoxia condition (YPR, 5 % DO)	6.0	8	42
	Hyper-oxygenation condition (YPR, 400 % DO)	6.5	9	9
High-Five cells	**Standard conditions: YPR** (50 % DO)	**4.0**	**4**	**6**
	Low glucose medium (50 % DO)	2.0	1	59
	Low Gln medium (50 % DO)	2.6	6	15
	Low Glucose and low Gln medium (50 % DO)	1.8	32	65
	Hypoxia condition (YPR, 5 % DO)	2.6	6	35
	Hyper-oxygenation condition (YPR, 400 % DO)	2.4	6	8

The influence of nutrient deprivation was examined using High-Five and Sf-9 cell batches inoculated into media of varying composition. For both cell lines, the maximal cell density decreased upon cultivation in low glucose (12 mM) or low Gln (4 mM) medium (Table 3-6) without any detection of more necrosis. Obviously, cells lacking glucose underwent apoptosis (Table 3-6 and Figure 3-3) but this was not the case with Gln-deprived medium (no DNA ladder seen).

The effects of hypoxia and of oxidative stress due to hyper-oxygenation on insect cell death were investigated using High-Five and Sf-9 cells cultivated in the bioreactor at a

dissolved oxygen concentration (DO) of 5% and 400 of air saturation. For both cell lines, the growth rate of cells cultivated under hypoxia conditions decreased, apoptosis markedly increased and necrosis rose weakly (Table 3-6). Upon hyper-oxygenation conditions, apoptosis and necrosis weakly increased.

3.4. DISCUSSION

3.4.1. COMPARISON OF METHODS

Different methods were used to characterize death in insect cells. They detected apoptosis at different stages. The AO/PI fluorometric assays detect the integrity of the plasma membrane and the compactness of the chromatin. The AO dye binds to RNA and DNA, single- and double-stranded, whereas PI binds only to double-stranded DNA. Viable and early apoptotic cells retain intact their plasma membrane and thus expel PI by an active mechanism that prevents its accumulation inside the cells, whereas late apoptotic and necrotic cells, with damaged plasma and nuclear membranes, do not (Singh and Al-Rubeai, 1998). The AO/PI allows to distinguish early from late apoptosis (Figures 3-2, 3-4 and 3-6).

The Trypan Blue dye stains the cell protein content and colours necrotic cells unable to expel this dye out of the cells by active transport, whereas viable cells are translucent (Altman et al., 1993). Unfortunately, the Trypan Blue test is unable to distinguish viable from apoptotic insect cells (Figures 3-4 and 3-6). Trypan Blue exclusion was the only method tested able to detect cell membrane damage by excessive stress (necrosis and post-apoptosis), whereas PI and Eth dyes detected solely necrosis. Annexin-V bound to the FITC fluorochrome, has the ability to bind to the phosphatidylserine displayed outside the cells during apoptosis (Pigault et al., 1994; Trotter et al., 1995). The detection of apoptosis with this method coincides with the detection of early apoptotic cells using the AO/PI test (Figure 3-4). Unfortunately, the Annexin-V-FITC binds also to the intracellular phosphatidylserine in necrotic cells with damaged membranes (Trotter et al., 1995). Concerning the calcein Am/Eth assays, the distinction between viable and apoptotic cells is highly subjective and thus its utility for the detection of apoptosis remains questionable. The AO/PI, calcein AM/Eth, Annexin-V-FITC/PI and Trypan Blue assays detected the morphological modifications occurring during necrosis and apoptosis, the latter activated either by the mitochondrial or by the receptor pathway. Even if apoptosis is reversible at some early stage, when morphological modifications become visible, the phenomenon is irreversible. Thus the AO/PI or Annexin-V-FITC tests are a proof of the presence of apoptotic cells.

Rh123 is a cationic fluorochrome that has the ability to accumulate inside negatively charged mitochondria of viable cells. During apoptosis, the mitochondrial membrane potential decreases and Rh123 moves into the cytosol (Schopf et al., 1990), allowing to detect the apoptosis when it is activated only by the mitochondrial pathway. The use of PI with Rh123 allowed to distinguish between necrotic and apoptotic cells (Figure 3-4). However, the Rh123/PI fluorometric assay is prone to some subjectivity problems, as reported by Métivier et al. (1998) with Jurkat cells (a T-lymphoblastoid cell line).

The detection of DNA fragmentation (Figures 3-3 and 3-5) by either DNA ladder or ELISA detection of DNA fragments is an unambiguous hallmark of apoptosis. However, some apoptotic cells do not have these morphological characteristics (Al-Rubeai, 1998). Moreover, false negative results are often possible and have already been reported with Sf-9 cells (Meneses-Acosta et al., 2001).

The enzymatic detection of the activity of caspase-3 (or its homolog, e.g. DrICE in *Drosophila*) allows to confirm the presence of the first downstream effector caspase activated by either the receptor or the mitochondrial pathway. This effector caspase is the first irreversible, detectable step of apoptosis (Al-Rubeai, 1998). In practice, for unknown reasons, this method detected the apoptosis in insect cells only after the detection, by other methods, of morphological modifications that normally appear later. Like the fragmentation of DNA, the detection of caspase-3 could produce false negatives.

LDH release by the dead cells is also a good marker of the presence of cellular debris or apoptotic bodies in the media, but this test could not distinguish cell destruction by necrosis from that by apoptosis followed by post-apoptotic necrosis (Figure 3-6). Nevertheless, this method is widely used to estimate the amount of destroyed animal cells (Goergen et al., 1993).

Finally, in addition to the classic Trypan Blue dye exclusion that is used to estimate cell density, any one among the AO/PI, Rh123/PI or Annexin-V-FITC/PI fluorometric assays is recommended to easily detect apoptosis in insect cells and to estimate the percentages of each cell type during cultivation. Although these fluorometric assays take longer than the Trypan Blue dye exclusion test, they are not any more complex or subjective for a trained operator and could be used daily.

Due to their poor capacity for quantification, their lengthy experimentation time and the possible detection of false negatives, the DNA fragmentation tests (DNA ladder and ELISA) and the detection of an effector-type caspase must be used only to confirm the findings of the previous methods.

A summary of all methods already used to detect insect cell death is reported, together with their respective features, benefits and drawbacks in Table 3-8.

During batch cultivation, experiments (Figures 3-4 and 3-6) suggested that there was no conversion of apoptotic High-Five cells into necrotic cells. Few necrotic cells were detected during the whole period of cultivation. Cell death by necrosis or apoptosis seems to result from two parallel mechanisms occurring simultaneity in the same batch. Thus, an early apoptotic cell would not become a necrotic cell in all cases. Dead cells were detected by the LDH assay from the first day of cultivation, which suggests that viable, early and late apoptotic and necrotic cells could get lysed. This cell death pathway is represented in Figure 3-7.

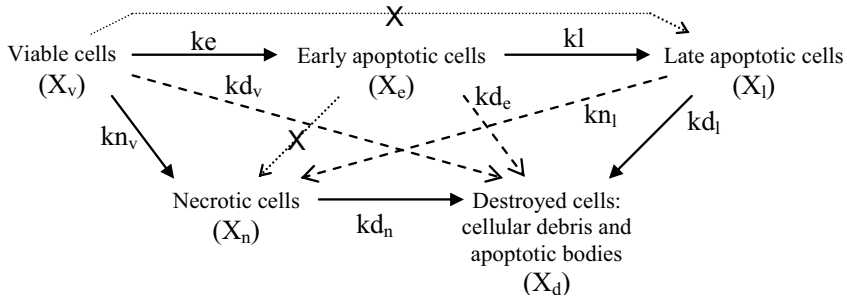

Figure 3-7: Stages of cell death. Cell species are distinguished by their appearance under fluorescence microscopy by AO/PI test, the cellular debris are estimated by LDH release. Full lines are widespread pathway of cell death, dashed lines are limited pathways, and crossed lines are pathways not occurring in insect cells

In anchorage-dependent cultivation, apoptosis was more marked in detached cells than in adherent cells. Thus, one of the morphological modifications brought about by apoptosis in insect cells was the loss of cell adhesion.

3.4.2. PHYSICAL FACTORS AFFECTING CELL DEATH

The short term effect of raising the temperature caused a decrease of viability on both High-Five and Sf-9 cell lines (Table 3-1). The increase of cultivation temperature from 22 to 30°C is known to increase the growth rate and the consumption of oxygen and glucose by Sf-9 cells (Reuveny et al, 1993a). But cultivation at 35°C decreases these parameters. Increasing the temperature from 22 to 27°C is also known to increase the production of β-gal using the BEVS, but with temperatures higher than 27°C, the productivity decreases (Reuveny et al, 1993b). When cells were adapted to long term passaging at high temperature, the growth rate, the protein synthesis and the membrane fluidity of insect cells decreased and cells produced heat shock proteins (HSP) (Gerbal et al., 2000). The Sf-9 cells are known to be more thermo-tolerant than High-Five cells, and begin producing HSP

proteins at 43°C. A clone of Sf-9 (SF9-HT) was shown to be capable of growth at 37°C after long-term passaging at this temperature (Gerbal et al., 2000).

As for shear stress, the absence of Pluronic F-68 caused a lethal stress by necrosis of all the High-Five population within 2 days. The decrease of the concentration of this copolymer, usually required to protect the cells from shear stress (Murhammer and Goochee, 1990), caused an increase in the number of apoptotic cells (Table 3-2). On the contrary, the increase of agitation rate from 130 rpm to 200 rpm caused an increase of cell density attained, probably due to the increase of oxygen transfer rate and to the tolerance of High-Five cells to the temporary increase of average shear stress. However, under intense agitation > 200 rpm, apoptosis increased (data not shown), as has already been observed in hybridoma cultivation (500-1600 rpm, Al-Rubeai et al., 1994).

The entirety of these chemical and physical factors causing insect cell death in suspension cultivation is recapitulated in Table 3-7.

Table 3-7: Summary of the insect cell suspension culture conditions inducing cell death

Environmental conditions		Apoptosis	Necrosis
Metabolic factors	Serum deprivation	Very high	Moderate
	Extract deprivation	Very high	Moderate
	Glucose deprivation	Very high	Low
	Gln deprivation	Low	Very low
	Low oxygen	Very high	Low
	High oxygen	Moderate	Low
	High ammonia concentration	High	Very low
	High lactate concentration	Moderate	Very low
Physical and chemical factors	Moderate low pH	Moderate	Very low
	Moderate high pH	Moderate	Very low
	Moderate level of hydrodynamic stress	Moderate	High
	High level of hydrodynamic stress	High	Very high
	Absence of Pluronic F-68	Low	Very high
	Cultivation at high temperature	Very high	Low
	Cultivation at low temperature	High	Moderate
	Cryopreservation	High	Very low

3.4.3. METABOLIC FACTORS AFFECTING CELL DEATH

The presence of the extracts Yeastolate and Primatone increased growth rate and cell density. Moreover, they seemed to have anti-apoptotic effects (Table 3-3). Yeastolate, which contains amino acids, peptides and B vitamins, has a growth promoting effect (Bédard et al., 1994; Vaughn and Fan, 1997), whereas Primatone is already known to have an anti-apoptotic effect (Schlaeger, 1996) and to extend the stationary phase of insect cell

growth (Donaldson and Shuler, 1998; Ikonomou et al., 2001). The presence of Yeastolate in the media was recently identified as essential for the production of some autocrine growth promoting factors by Sf-9 cells (Calles et al., 2006). Furthermore, these extracts (as well as serum) have also surfactant properties. The anti-apoptotic and growth promoting influence of these extracts could be due to nutritional effects, to the presence of bioactive substances, etc. Furthermore, their high antioxidant capacity (Table 3-4) could be an additional source of their beneficial effects on insect cell cultivation.

The deprivation of any single amino acid in hybridoma culture leads to apoptotic death (Simpson et al., 1998). In Sf-9 insect cells, Arg, Cys, His, Ile, Leu, Lys Met, Phe, Pro, Val, Thr, Trp, Tyr are essential amino acids, and the lack of any one of them was found to stop growth (Ferrance et al., 1993; Radford et al., 1997). Nevertheless, the lack of Gln seemed to trigger neither cell apoptosis nor necrosis (Figure 3-3), although a decrease of the maximal High-Five and Sf-9 cell density was observed. On the contrary, when these cells lacked glucose, a high degree of apoptosis was observed (Table 3-6). The activation of apoptosis in High-Five cells coincided in time with the decline of viability, the arrest of growth and the limitation of glucose (Table 3-6 and Figure 3-3) as already observed with Sf-9 cells by Meneses-Acosta et al. (2001).

Hypoxia, as previously noted by Mercille and Massie (1994) on hydridoma cells, strongly activated the apoptosis of our insect cells, probably due to the insufficiency of oxygen in its role as a nutrient (Table 3-6). On the contrary, hyper-oxygenation, an instance of oxidative stress, seemed to only weakly activate apoptosis in High Five and Sf-9 cells (Table 3-6), whereas higher oxidative stresses such as UV irradiation or the presence of H_2O_2 are known to strongly activate apoptosis in Sf-9 cells (Hasnain et al., 1999).

The presence of lactate diminished growth rate and maximal High-Five cell density and primed the further production of lactate, but did not activate apoptosis (Table 3-5), as previously seen (Drugmand et al., 2005). The presence of high concentration of lactate in media is already known to stimulate death by necrosis in hydridoma and myeloma cells (Mercille and Massie, 1994; Singh et al., 1994) and also in insect cells (Singh et al., 1994; Drugmand et al., 2005). The toxicity of lactate to insect cells is probably similar to mammalian cells (BHK), due to medium acidification and the increase of osmolarity (Cruz et al., 2000).

On the contrary, the presence of high ammonia concentration activated the apoptosis of our High-Five insect cells (Table 3-5) as previously observed in hydridoma cells by Singh et al. (1994). Although the export of ammonia out of the cells triggers an increase of extracellular pH, this medium basification did not appear to activate apoptosis (Table 3-5).

This suggests that the ammonia molecule itself may be toxic for insect cells. Even if its toxicity effect could not be clearly explained, it probably results, as in mammalian cells, from the acidification of the mitochondria and cytoplasm (due to the equilibrium $H_2O + NH_3 \leftrightarrow NH_4^+ + OH^-$) and the modifications of K^+ gradient due to the competition of K^+ with the ammonium cation for the Na^+/K^+-ATPase transporters (Martinelle et al., 1996; Schneider et al., 1996).

Hence, upon lack of glucose or presence of ammonia, apoptosis could be detected using the Rh123 test, which picks up the loss of the mitochondrial membrane potential. Thus, these metabolic factors probably activate apoptosis by the mitochondrial pathway. The factors causing death in uninfected insect cell suspension cultivation are summarized in Table 3-7.

3.5. CONCLUSION

Amongst the various methods compared, the microscopic fluorometric methods (AO/PI, Rh123/PI or Annexin-V-FITC/PI) allow to quantify apoptosis and to classify cell types during cultivation. However, these methods could be subjective. Thus the use of qualitative methods (DNA fragmentation, detection of caspase, etc.), which are less subjective, is advised to confirm the presence of insect cell apoptosis during different phases of bioprocessing cultivation (Table 3-8).

The various relatively moderate chemical and physical stresses tested (variation of temperature, agitation, concentration of Pluronic F-68, change of pH) with Sf-9 and High-Five cells triggered a shortening of the growth phase, but did not seem to affect the apoptosis of these insect cells.

On the contrary, the moderate metabolic stresses tested (lack of glucose, Gln or hydrolysates, presence of metabolic by-products) clearly induced apoptosis in High-Five insect cells. Specifically for cultured insect cells (High-Five and Sf-9), the metabolic factors that trigger apoptosis are the lack of glucose and the production of ammonia.

Table 3-8 (next page): Summary of the methods used to detect insect cell death.

[1]: this work, [2]: Clem and Miller (1993), [3]: Clem and Miller (1994), [4]: Singh et al. (1994) staining with AO without PI, [5]: Chejanovsky and Gershburg (1995) staining with DAPI, [6]: Du and Thiem (1997), [7]: Lacount and Friesen (1997), [8]: Cowger et al. (1999), [9]: Dai et al. (1999), [10]: Du et al. (1999), [11]: Hasnain et al. (1999), [12]: Zoog et al. (1999) caspase-3, [13]: Rhee and Park (2000) [14]: Gotoh and Kikuchi (2001), [15]: Meneses-Acosta et al. (2001) staining with AO and ethidium bromide, [16]: Pei et al. (2002) caspase-1, [17]: Zhang et al. (2002), [18]: Fornelli et al. (2004).

Methods	Principle	Detected characteristics	Quantitative
Dyes			
Trypan Blue	Dye exclusion Optical microscopy or automated counter	Integrity of the plasma membrane	Yes
AO / PI	Fluorescent dye Fluorescent microscopy or flux cytometry	Integrity of the plasma membrane Compactness of the chromatin	Yes
Rh 123 (with PI)	Fluorescent dye Fluorescent microscopy or flux cytometry	Integrity of the plasma membrane Change in the mitochondrial potential	Yes
Calcein AM/ Eth	Fluorescent dye Fluorescent microscopy, flux cytometry or automated counter	Integrity of the plasma membrane	Yes
Annexin-V-FITC (with PI)	Fluorescent dye Fluorescent microscopy or flux cytometry	Integrity of the plasma membrane Flipping of phosphatidylserine	Yes
DNA fragmentation			
Test TUNEL (Terminal transferase Utilizing Nick End Labelling)	ELISA test Flux cytometry (antibodies coupled with fluorochromes)	Detection of fragment of DNA by antibodies (after incorporation)	Weak
DNA ladder	Electrophoresis	Detection of DNA fragment multiples of 180 bp	No
Proteins detection			
Detection of caspase	Spectroscopic of fluorometric enzymatic assay	Detection of enzymatic activity	Yes
Cleavage of protein	Westren blot (Dot blot) or Flux cytometry[4]	Clivage of PARP or BID by caspases	No
LDH test	Spectroscopic enzymatic assay	Detection of the cytosolic LDH enzyme into supernatant	Yes
RNA detection			
RT-PCR	RT-PCR Real-Time (RT) PCR	Detection of transcription of a apoptosis gene (usually gene p35 of AcMNPV during infection)	Weak
Cellular analysis			
Electronic microscopy	Electronic microscopy Cell morphology analysis	Compactness of the nuclei, Detection of apoptotic bodies Integrity of the plasma membrane	No
Flux cytometry	Flux cytometry Diffusion of light accros the cells Leak of DNA out of the cells DNA histogram	Compactness of the nuclei, Detection of apoptotic bodies Integrity of the plasma membrane Used with or without fluorescent dyes	Yes

Methods	Benefits	Drawbacks	Detection of cell death	Insect Cell lines
Trypan Blue	Easy, simple, cheap	Subjectivity Cytotoxicity of the dye Imprecise	Only necrosis	High-Five [1] Sf-9 [1, 11, 15]
AO / PI	Easy, simple Differentiation of early, late apoptosis and necrosis (only with microscopy)	High subjectivity Cytotoxicity of the dye Imprecise	Early and late apoptosis, Necrosis	High-Five [1] Sf-9 [1, 4, 9, 15] Sf-21 [5] SL-2 [5]
Rh 123 (with PI)	Differentiation of necrosis and apoptosis (only activate by the mitochondrial way)	Subjectivity Cytotoxicity of the dye Imprecise Possibility of false positive	Apoptosis Necrosis	High-Five [1] Sf-9 [1]
Calcein AM/ Eth	Easy, simple Cheap	High subjectivity to detected apoptosis Cytotoxicity of the dye Imprecise	Only necrosis	High-Five [1] Sf-9 [1]
Annexin-V-FITC (with PI)	High sensitivity	Over-estimation of the apoptosis Dye very expensive	Early apoptosis Necrosis	High-Five [1] Sf-9 [1]
DNA fragmentation				
Test TUNEL (Terminal transferase Utilizing Nick End Labelling)	Conservation of withdraw samples	Long Costly Very low sensibility for insect cells False negative	Apoptosis	High-Five [1] Sf-9 [1, 13, 17]
DNA ladder	Indisputable criteria (for the positive)	Long Possibility of false negatives Purification False negative	Apoptosis	High-Five [1, 9] Sf-9 [1, 4, 5, 11, 14, 15, 17] SF-21 [2, 6, 7] Tn-386 [2, 7] SL-2 [5]
Proteins detection				
Detection of caspase	Sensible Most universal than an ELISA test	Costly Detection of mammalian caspases False negative	Apoptosis	High-Five [1] SF-9 [16] SF-21 [12] Tn-386 [16]
Cleavage of protein	Sensible	Long Specific antibodies	Apoptosis (mitochondrial or receptor way)	SF-9 [5, 10] SF-21 [3, 6] Tn-386 [3] SL-2 [5] Ld652Y [6]
LDH test	Fast Efficient Sensible	Weak conservation of the samples	Only necrosis	High-Five [1] Sf-9 [1]
RNA detection				
RT-PCR	Flexible	Need knowledge on the gene or proteins implicated Costly Long	Apoptosis	High-Five [9] SF-21 [2, 3] Tn-386 [2, 3] Ld652Y [6]
Cellular analysis				
Electronic microscopy	High sensibility Lot of information on cells	Very expensive Very long Not usable daily Difficult to apply	Early and late apoptosis Necrosis	Sf-9 [4, 15, 17]
Flux cytometry	Reproducible Efficient	Costly Various problem with polyploidy Confusion possible between apoptotic and necrotic cells	Apoptosis Necrosis	SF-9 [9, 15, 18] SF-21 [3] Tn-386 [3]

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4

PART II: INSECT CELL PHYSIOLOGY AND METABOLISM

Chapter 4 : Analysis of the metabolism of lepidopteran cell lines using a metabolic network

4.1. INTRODUCTION

In the last two decades, the Insect Cell - Baculovirus Expression Vector System (IC-BEVS) has become popular for producing numerous recombinant (glyco-)proteins. The simplicity of insect cell cultivation in serum-free media and the easy construction of a wide variety of recombinant baculovirus vectors have made the IC-BEVS quite an effective transient expression system. Among insect cell lines, *Trichoplusia ni* High-Five™ and *Spodoptera frugiperda* Sf-9 cells have a great potential for the production of recombinant proteins using the BEVS system, as they are able to grow in suspension in serum-free media at high agitation rate, to attain high cell densities and to reach high protein production levels. Since the development of insect cell cultivation, various pathways of the in-vitro metabolism of lepidopteran cells have been established. Most of these studies address Sf-9 metabolism and only a few consider the metabolism of High-Five cells.

In this study, we have used a metabolic flux network to analyze the catabolism and anabolism of High-Five and Sf-9 cells under various culture conditions during the growth and infection phases. Such flux analyses have already been used previously to quantitatively describe pathways in mammalian cells (Xie and Wang 1996a; Xie and Wang 1996b) but only one attempt has been made with insect cells, and this only to study a portion of the catabolism of Sf-9 cells (Ferrance et al. 1993). The value of such models lies in the possibility to determine the intracellular patterns of metabolism, but they require the determination of the enzymatic pathways and the identification of the critical junctions (or nodes) in the network (Stephanopoulos and Sinskey 1993).

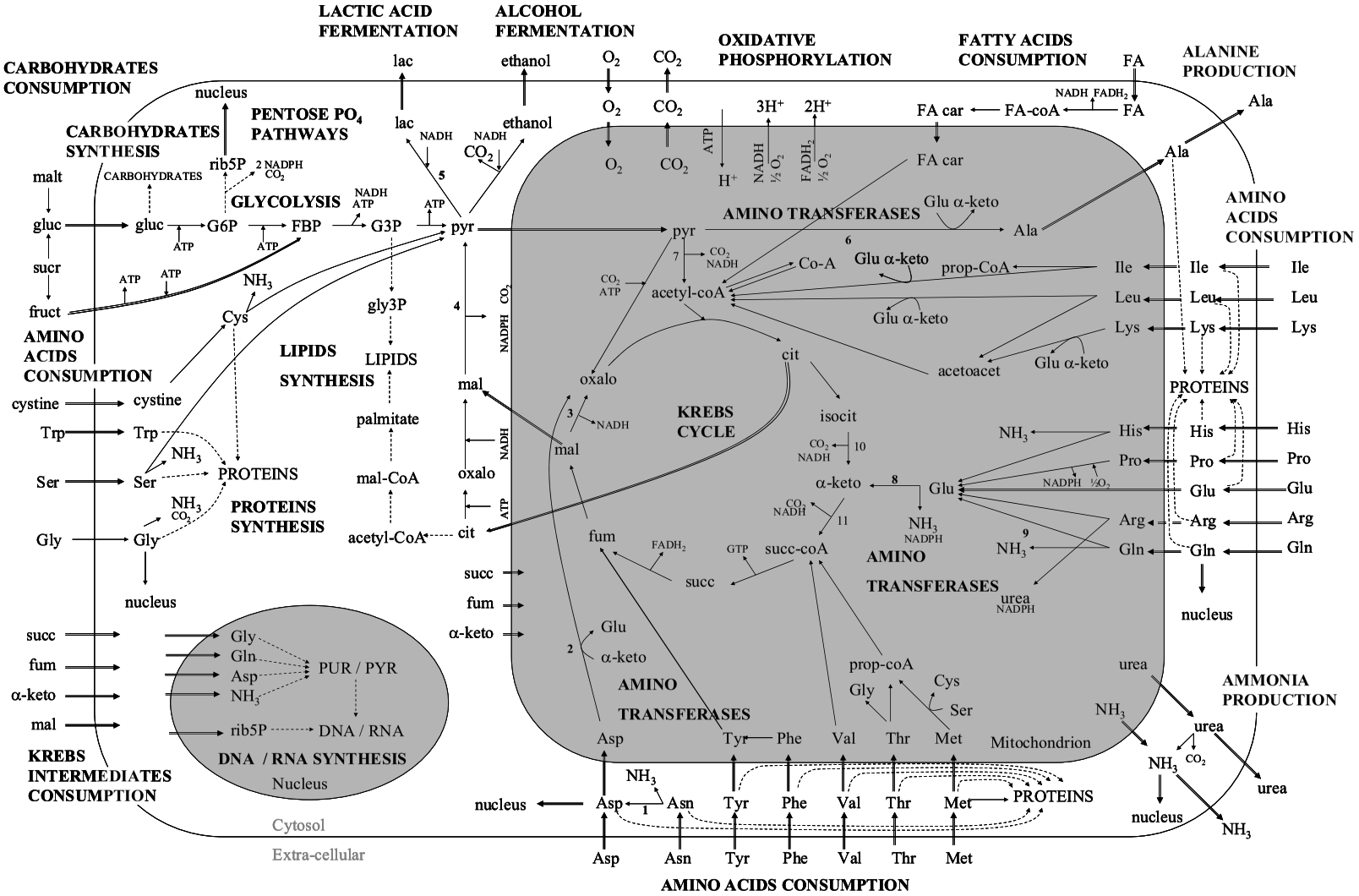
Metabolic flux network

Flux analyses could be useful to study a complex metabolic network even if a sufficient number of accurate measurements are available (Xie and Wang 1996a and b). The metabolic network involves fluxes (normalized flows of the metabolic pathways) and nodes (connections between metabolic pathways). All concentrations of all intracellular intermediates are assumed to be constant. Each flux represents one term that may be known or unknown (extracellular compounds or intracellular products that form cellular material during growth). Each node is expressed as an equation that involves various fluxes. The fluxes could be reversible or irreversible as a function of the enzymes present in the cells and irreversible fluxes imply inequality constraints. For one node, the sum of incoming fluxes must be equal to the sum of outgoing fluxes (Zupke and Stephanopoulos 1994).

The system is set up in matrix form (Equation 4-1). **V** is the vector of fluxes (v_1, …,v_p) where *p* is the number of fluxes. **A** is a *(m x p)*-matrix of node equations where a_{11},…, a_{1p}, a_{m1},…a_{mp} are their respective stoichiometric coefficients. **B** is a (*t x p*)-matrix of constraint equations where b_{11},…, b_{1p}, b_{t1},…b_{tp} are their respective stoichiometric coefficients. *m* and *t* are respectively the number of non redundant node equations and constraint equations. **C** is the vector of values of known fluxes (c_1,…, c_n) where *n* is the number of known fluxes. Hence, *p-n* is the number of unknown fluxes. To solve the system, the number of non redundant node equations (*m*) and constraint equations (*t*) must be equal to the number (*p*) of total fluxes: $\boldsymbol{m + t = p}$. Moreover, the number of total fluxes (*p*) minus the number non redundant node equations (*m*) must be equal to the number (*n*) of known fluxes: $\boldsymbol{p - m = n}$. **D** (*m+t x p*)-matrix , **E** (*1 x p*)-matrix and **F** (*1 x m+n*)-matrix (Equation 4-1) are matrix used to solve the equation system.

$$\begin{matrix} \text{node} \quad \text{equation} \\ \\ \text{constraint} \quad \text{equation} \end{matrix} \underbrace{\begin{bmatrix} a_{11} & \cdots & a_{1p} \\ \vdots & \cdots & \vdots \\ a_{m1} & \cdots & a_{mp} \\ \hline b_{11} & \cdots & b_{1p} \\ \vdots & \cdots & \vdots \\ b_{t1} & \cdots & b_{tp} \end{bmatrix}}_{Matrix\ D} \underbrace{\begin{bmatrix} v_1 \\ \vdots \\ \vdots \\ \vdots \\ \vdots \\ v_p \end{bmatrix}}_{Matrix\ E} = \underbrace{\begin{bmatrix} 0 \\ \vdots \\ 0 \\ \hline c_1 \\ \vdots \\ c_n \end{bmatrix}}_{Matrix\ F} \qquad \text{(Equation 4-1)}$$

Figure 4-1 (next page): Metabolic pathways in a generic insect cell used for the construction of the metabolic flux network. Full lines, dashed lines and double lines represent, respectively, the catabolism pathways, the anabolism pathways and the transfer of compounds from one cellular compartment to another. Enzymes : 1, asparaginase; 2 aspartate aminotransferase, ; 3, malate dehydrogenase; 4, malic enzyme ; 5, lactate dehydrogenase; 6, alanine aminotransferase; 7, pyruvate dehydrogenase; 8, glutamate dehydrogenase; 9, glutaminase; 10, isocitrate dehydrogenase; 11, α-ketoglutarate dehydrogenase. Abbreviations: α ketoglutarate, acetoacet: acetoacetate, cit: ci trate, FA: fatty acid, FA-car: fatty acid carnitine, FA-CoA: fatty acid-CoA, FBP: fructose-1,6-bisphosphate, fruct: fructose, fum: fumarate, G3P: 3-phosphoglycerate, G6P: gl ucose-6-phosphate, gluc: glucose, gly3P: glycerol-3-phosphate, isocit: isocitrate, lac: lactate, mal: malate, mal-CoA: malonyl-CoA, malt: maltose, oxalo: oxaloacetate, prop-CoA: propionyl-CoA, PUR: purines, PYR: pyrimidines, pyr: pyruvate, rib5P: ribose-5-phosphate, succ: succinate, succ-CoA: succinyl-CoA, sucr: sucrose


LACTIC ACID FERMENTATION
ALCOHOL FERMENTATION
OXIDATIVE PHOSPHORYLATION
FATTY ACIDS CONSUMPTION
ALANINE PRODUCTION
CARBOHYDRATES CONSUMPTION
CARBOHYDRATES SYNTHESIS
PENTOSE PO4 PATHWAYS
GLYCOLYSIS
AMINO ACIDS CONSUMPTION
LIPIDS SYNTHESIS
PROTEINS SYNTHESIS
KREBS CYCLE
AMINO TRANSFERASES
AMMONIA PRODUCTION
KREBS INTERMEDIATES CONSUMPTION
DNA / RNA SYNTHESIS
Nucleus
Cytosol
Extra-cellular
Mitochondrion
lac
ethanol
nucleus
malt
gluc
sucr
fruct
rib5P
G6P
FBP
G3P
pyr
Cys
gly3P
palmitate
mal-CoA
acetyl-CoA
oxalo
mal
cit
isocit
α-keto
Glu
succ-coA
succ
fum
prop-coA
acetoacet
Co-A
cystine
Trp
Ser
Gly
PROTEINS
Ala
Ile
Leu
Lys
His
Pro
Arg
Gln
Asp
Asn
Met
Thr
Val
Phe
Tyr
PUR / PYR
DNA / RNA
urea
FA
FA-coA
FA car

Metabolic pathways

The major key to elaborate the metabolic flux network is structuring the existing metabolic knowledge in order to analyze the metabolism quantitatively. This methodology requires use of a correct pathway network and knowledge of the stoichiometry of the enzymatic reactions involved. In Sf-9 and High-Five insect cells the majority of their pathways is in common with other eukaryotic cells but they lack some enzymes expressed in mammalian cells. Moreover, insect cells cultivated in vitro are known to have some pathway flows distinct from mammalian cells. The network shown in Figure 4-1 is built on prior knowledge from the literature and includes three types of fluxes: catabolic pathways (full lines), anabolic pathways (dashed lines) and fluxes of metabolite transfer from one cellular compartment to another (double lines).

The main nutrient of in vitro cultivated insect cells is glucose that enters the cell *via* a glucose transporter (Murray et al. 1996) and sustains the glycolysis forming pyruvate (Figure 4-1). However, insect cells can also use maltose, fructose and sucrose as alternatives under particular conditions, following the hydrolysis of these dimeric sugars outside the cell (Bédard et al. 1993).

Pyruvate enters the mitochondrion and feeds into the Krebs cycle which is common to all eukaryotic cells (Gilmour 1961; Lehninger et al. 1993; Murray et al. 1996; Voet and Voet 2005). However, glucose can also be used for carbohydrate synthesis. Glucose-6-phosphate (G6P) and 3-phosphoglycerate (G3P) are used respectively in the pentose phosphate pathway to produce ribose-5-phosphate (rib5P) required for the formation of purine and pyrimidine nucleotides and for the production of glycerol-3-phosphate (gly3P), which in turn is used to synthesize triglycero-, glycero- and phospholipids (Ferrance et al. 1993; Gilmour 1961; Murray et al. 1996; Öhman et al. 1995).

The Krebs cycle produces CO_2 and provides NADH and $FADH_2$ for the oxidative phosphorylation process. Oxygen is used to generate a proton gradient, necessary for the production of ATP as energy currency. For the construction of our network, we used stoichiometric reactions for CO_2 production in the Krebs cycle, for oxygen consumption and for ATP production via oxidative phosphorylation (Murray et al. 1996). Pyruvate can also be reduced to lactic acid in the cytosol, as in the majority of mammalian cell lines, but insect cells can also produce ethanol as proposed by Takahashi et al. (1995) and Doverskog et al. (2000a).

In parallel with the Krebs cycle, insect cells convert pyruvate to oxaloacetate (Murray et al. 1996), malate to pyruvate (Bédard et al. 1993; Ferrance et al. 1993), and use amino acids in anaplerotic pathways to feed into the Krebs cycle (Ferrance et al. 1993;

Neermann and Wagner 1996; Öhman et al. 1995). Alanine aminotransferase converts Glu + pyruvate to Ala + α–ketoglutarate inside the mitochondrion (Gilmour 1961; Koval et al. 1976). This transaminase in contrast with glutamate dehydrogenase prevents the production of toxic NH_3 (Öhman et al. 1995). Although Öhman et al. (1995) proposed that the conversion of pyruvate to Ala is done in the cytosol, we decided to follow the hypothesis of Ferrance et al. (1993) who placed this enzyme in the mitochondrion, which is more in tune with other eukaryotic cells (Murray et al. 1996).

The excess of malate and citrate produced by the Krebs cycle can be exported out of the mitochondrion to produce pyruvate or lipids (Lehninger et al. 1993; Murray et al. 1996). The insect cells are also able to consume the organic acids of the Krebs cycle (Ferrance et al. 1993) and to use lipids for the production of energy (Luukkonen et al. 1973; Murray et al. 1996).

Insect cells consume the majority of the amino acids for the production of proteins, purines and pyrimidines (in the case of Asp, Gln and Gly), but the cells degrade some of them to produce energy. Among the amino acids, the most important are the pairs Gln/Glu and Asn/Asp. Glutaminase converts Gln into Glu inside the mitochondrion. Glu is converted to α–ketoglutarate by glutamate dehydrogenase (Drews et al. 2000; Wang et al. 1993). This pathway is reversible in Sf-9 lepidopteran cells that possess a glutamine synthetase (Doverskog et al. 2000a and 2000b; Drew et al. 2000). Insect cells can also convert Arg, His and Pro to Glu (Bhatia et al. 1997; Öhman et al. 1996). The use of Arg in the Krebs cycle, after its conversion into glutamate, leads to the production of urea which is excreted in the supernatant without recycling into the urea cycle (Murray et al. 1996; Wyatt 1956).

Insect cells also consume Asn as energy source. Asn is converted to Asp in the cytosol, and the latter is transformed to oxaloacetate in the mitochondrion. Ile, Leu and Lys can be, directly or indirectly, converted into acetyl-coA (Murray et al. 1996; Xie and Wang 1996a; Xie and Wang 1996b). Met, Phe, Thr and Tyr enter the Krebs cycle after conversion into fumarate or succinyl-CoA (Bhatia et al. 1997; Doverskog et al. 1997). Ser and cystine/Cys can be converted to pyruvate in the cytosol (Bhatia et al. 1997; Doverskog et al. 1998; Doverskog et al. 1997; Radford et al. 1997). Ser could be also produced from Gly (Bhatia et al. 1997). Sf-9 cells are believed to be auxotrophic on Gly and Cys (Tremblay et al. 1992). Trp seems to be used only to synthesise proteins. Arg, Cys, His, Met, Phe, Pro, Val, Trp, and Tyr are required for growth of the EPa insect cell line according to Landureau (1969) and, Landureau and Jollès (1969). Ferrance et al. (1993) and Radford et al. (1997) found that the Ile, Lys, His, Leu and Thr are also essential for Sf-9

cells, whereas Ile, Lys, Thr, Ser are required in the growth phase according to Landureau and Jollès (1969). A lack of one of them brings growth to a stop. The presence of Met and Tyr is known to delay cell death in Sf-9 cultivation (Mendonça et al. 1999). Ala, Asn and Gln are required by the EPa cells to have a high growth rate (Landureau and Jollès 1969) and Arg, Asn, Asp, Gln, Glu, Gly and Ser by Sf-9 (Ferrance et al. 1993; Rhiel et al.1997) and by High-Five cells (Rhiel et al. 1997; Wang et al. 1993). Radford et al. (1997) have shown that these latter amino acids (Arg, Asn, Asp, Gln, Glu, Gly, Ser) are also required during the infection phase to obtain high production rates.

4.2. MATERIALS AND METHODS

4.2.1. CELL LINES AND VIRUS

The High-Five™ and Sf-9 insect cell lines were used. The High-Five cells were a gift of the Laboratory of Virology (University of Wageningen, The Netherlands), whereas Sf-9 were bought from Invitrogen (Carlsbad, CA).

The cells were adapted to suspension and maintained in exponential phase at 27°C and 130 rpm in shake-flasks (Corning, Acton, MA) of 125 ml with 25 ml of medium. Batch cultures of High-Five and Sf-9 in 250 ml shake-flasks (50 ml of medium) were used for the study of uninfected cells. Cells were inoculated at 0.2 and $0.4x10^6$ cells/ml, respectively, for High-Five and Sf-9 in YPR medium (Ikonomou et al. 2001) to which 67 kDa dextran (1g/L) was added.

Cells were infected with an *Autographa californica* (AcMNPV) baculovirus encoding β-galactosidase (β-gal) (Invitrogen) at 4 and 8.10^6 cells/ml, respectively, for High-Five and Sf-9 cells (250 ml shake-flasks, 50 ml of medium, MOI of 2), after a total change-over of medium by centrifugation (50 x g, 10 min). Supernatant samples were withdrawn daily (1 ml) to measure the cell density as well as the consumption of metabolites and the accumulation of metabolic by-products. The cell densities and viability were estimated with a Neubauer hemocytometer using Trypan Blue dye exclusion (0.2% w/v final concentration). Supernatants were centrifuged (1000 x g, 10 min) before metabolite analysis. The produced β-gal was assayed according to Miller (1972).

4.2.2. NUTRIENT AND BY-PRODUCT ASSAYS

Analyses of glucose, lactic acid, ammonia and pH were done using a Bioprofile 100 Analyser (Nova Biomedical, Waltham, MA). Gln and Glu were determined by a Glu enzymatic kit (Boehringer, Mannheim, Germany). Glu and Gln samples were analysed separately. Before analysis, Gln samples (50 µl) were treated with 10 µl asparaginase

(400 units/ml) (Sigma) in order to transform completely Gln to Glu (37°C, 30 min in PBS buffer), then the sum of Glu plus Gln were determined. Gln was determined by subtraction of the previously determined Glu.

The other free amino acids (Ala, Arg, Asn, Asp, Cys, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Tyr, Val) in the samples were analysed by high-pressure liquid chromatography (HPLC) after deproteinization with sulfosalicylic acid and *o*-phthaldialdehyde (OPA) derivatization using a Spherisorb ODS column (Waters, Brussels, Belgium) as described by Fekkes et al. (1995).

The total amino acids consumption (free amino acids plus amino acids present in hydrolysates) was determined by the Fekkes HPLC method after total peptide hydrolysis with 6 M hydrochloric acid for about 24 hours at 110°C and neutralisation of the acid with NaOH. Maltose, sucrose and fructose were tested using Maltose/Sucrose/D-Glucose and D-Glucose/D-Fructose Boerhinger assays. The urea and ethanol by-products and the L-malic acid, succinic and fumaric organics acids were measured using the urea/NH_3 test and the respective Boerhinger assays. The lipids consumed by the cells were measured by a modification of the classical Soxhlet method extraction of total lipids by ether after dehydration of the sample and hydrolysis by HCl (Manirakiza et al. 2001). The consumption of oxygen and the production of CO_2 by cells were estimated by dynamic measurements during the exponential phase in bioreactor cultivation.

4.2.3. Cell content estimation

To determine cell biomass content, four shake-flasks of 500 ml were inoculated with $0.4.10^6$ cells/ml of High-Five and Sf-9 and are cultivated in YPR medium. When cells reached ca. 4.10^6 cells, the cell densities were measured by Trypan Blue exclusion (5 determinations) and two shake-flasks were infected at a MOI of 2. The two others were centrifuged (20 min, 500 x g) and the pellets were withdrawn. After 48 hpi, the cell densities of the infected flasks were measured and the flasks centrifuged. The pellets were rinsed three times with PBS and used for the determination of proteins, DNA, RNA, lipids and carbohydrates, normalized to the total numbers of cells.

The protein, DNA and carbohydrate contents of the cell biomass were estimated, respectively, with the Bradford assay (Bio-Rad Laboratories, Hercules, CA), a modification of the Hoechst test (Labarca and Paigen 1980) and the anthrone test (Jermyn 1975). The quantity of RNA was estimated by a "standard-addition method" (addition of a known quantity of RNA to the sample) and measurement of the absorbance at 260 nm after lysis in Tri-instapure and separation/purification by a chloroform/ethanol/isopropanol method as described in Berger et al. (2003). The mass of lipids in the pellet was measured by a

modification of the classic Soxhlet method after dehydration and hydrolysis by HCl (Manirakiza et al. 2001).

4.2.4. RADIOACTIVE LABELLING EXPERIMENTS

The fate of the Asn in High-Five cells was investigated by adding 1 µCi/ml of L-[^{14}C(U)]Asn (American Radiolabeled Chemicals, Saint-Louis, MO) to a culture containing 2.10^6 High-Five cells in 1 ml, placed into a sterile headspace vial tube at 27°C at 130 rpm for 36 hours. At the end of the incubation, the media were colleted and the cells centrifuged (10 min, 100 x g), and then rinsed twice with PBS. The cells in the pellet were lysed using 1 ml of Triton X-100 1g/L (Sigma).

The amounts of Ala and lactate produced and the residual radiolabeled Asn and glucose were separated by TLC (silica gel thin layer, Merck, Whitehouse Station, NJ) with an acetone/water/chloroform/ethanol/ammonium hydroxide solution (28%) (60:2:6:10:22), and radiographed on a ECL hyperfilm (Amersham, Buckinghamshire, UK). The spots of Ala, Asn and Asp were developed by spraying ninhydrine (1 % in acetone) whereas the spots of lactic acid and glucose standards were developed with a mixture of red methyl and bromophenol (2.5 mg/ml in 70 % methanol, Lee et al. 2001). The labelled spots followed were scraped and analysed by liquid scintillation spectrometry (Packard TriCarb 1600 TR, Packard Instrument Company, Meriden, CT) after dispersion in 5 ml of Aqualuma cocktail (Luma LSC, Groningen, The Netherlands).

The accumulation of ^{14}C into the cell biomass was estimated by measuring the radioactivity of the pellet. The analysis of the release of the $^{14}CO_2$ was done in similar conditions, but after addition of a 100 µl of sterile 1 M hyamine in methanol, placed in the headspace vial during 8 hours. The tube was sealed after adding the Asn^{14}C. At the end of the incubation, the $^{14}CO_2$ released in the tubes is driven out of the media by addition with a syringe of 100 µl of 0.1 M Na_2CO_3 and 100 µl of HCl for 45 min at 27°C (Stell et al. 2001). The hyamine solution containing the $^{14}CO_2$ was diluted with 1 ml of methanol and 5 ml of Aqualuma cocktail and counted for radioactivity.

4.3. RESULTS

4.3.1. DEVELOPMENT OF THE METABOLIC FLUX NETWORK

In the metabolic network fluxes presented in Figure 4-1, there are 146 fluxes (*p*) and 86 nodes (*m*). Some of these fluxes are known (*n*), others are unknown (*p*-*n*). All of the fluxes used in the network were specific rates, obtained by dividing the volumetric consumption/production rate by the cell density. The experimental data are the macroscopic extracellular consumption/production of some metabolites, the growth rate of the cells and the intracellular content of protein, DNA/RNA, lipid and carbohydrate.

Extracellular known fluxes

During the entire cultivation, macroscopic specific rates (q) of consumption/production of metabolites (nutrients and by-products) were estimated ($q= Q / \mu$) on the basis of their volumetric consumption/production rates ($Q= \Delta M / \Delta t$) and the growth rate of (viable) cells ($\mu= \Delta X / \Delta t$) within an interval of time (Δt) of 24 h {where X is the viable cell density (10^6 cells/ml) and M is the metabolite (nutrients or by-products) concentration (µM) }. Specific consumption rates (µmol/10^9 cells.h) of carbohydrates (glucose, maltose, sucrose, fructose) (number of known fluxes *n* is 4), specific production rates of by-products (lactate, NH_3, urea and ethanol) (*n*=4+4=8), specific consumption rate of malic, fumaric, succinic acids (*n*=8+3=11) were followed by enzymatic assays, whereas specific consumption/production rates of the amino acids (except the Pro and Trp that are not-measurable by HPLC) were estimated by HPLC analysis (*n*=11+18=29). The specific consumption rate of total lipids was evaluated using the Soxhlet method (*n*=29+1=30). The average of these rates was used in the metabolic flux network: between 24 h and 96 h for the uninfected cells and between 0 hour post infection (hpi) and 96 hpi for the infected cells. The average consumption rate of α-ketoglutarate (*n*=29+1=30) present in the medium was evaluated from the quantity present in the medium and its consumption rate by Sf-9 and High-Five cells in IPL-41 medium, our basal medium (Bédard et al. 1993). At this stage, 31 fluxes are known.

Intracellular known fluxes

The cellular contents in protein, DNA, RNA, lipid and carbohydrate are listed in Table 4-1 for High-Five and Sf-9 cultivated in YPR medium. These data are close to the typical values found in the literature: lipid (Luukkonen et al. 1973; Vaughn 1973), carbohydrate (Davis et al. 1993), protein and DNA/RNA (Rosinsky et al. 2000).

Table 4-1: Insect cell biomass composition

Insect cell biomass composition		Uninfected cells		Infected cells	
		High-Five	Sf-9	High-Five	Sf-9
DNA content	$\mu g / 10^6$ cells	2.87 ± 0.42	2.10 ± 0.22	2.18 ± 0.21	1.89 ± 0.21
RNA content	$\mu g / 10^6$ cells	11.74 ± 1.66	7.95 ± 1.78	11.86 ± 1.34	11.35 ± 3.30
Total protein content	$mg / 10^6$ cells	1.12 ± 0.08	0.75 ± 0.08	1.32 ± 0.09	0.69 ± 0.08
Total lipids content	$\mu g / 10^6$ cells	80 ± 2	76 ± 2	79 ± 3	77 ± 2
Total carbohydrates content*	$\mu g / 10^6$ cells	24 ± 2	21 ± 2	26 ± 2	17 ± 3

*carbohydrates plus sugars present in glycoproteins

Table 4-2: Amino acid composition in invertebrate cell proteins

Amino acid	Occurrence (%)*
Ala	7.5
Arg	4.3
Asn	5.4
Asp	5.4
Cys	1.7
Gln	3.9
Glu	5.8
Gly	6.9
His	2.4
Isol	6.1
Leu	8.9
Lys	6.5
Met	2.0
Phe	4.4
Pro	4.6
Ser	7.1
Thr	5.8
Trp	1.4
Tyr	3.8
Val	6.1

*percents in w/w, data fo r invertebrate protein from Doolittle et al. (1986)

For the same organism, the entirety of proteins is composed of the same proportion of each amino acid (Doolittle et al. 1986). Based on the occurrence of the 20 amino acids present in invertebrate cells (Table 4-2) and on their molecular weight (MW), the average MW of one amino acid and the amount of each amino acid required to form one gram of protein (1 mg protein: 8.96×10^{-6} mol of an "average" amino acid of 111.6 g/mol) was evaluated. Therefore, based on the growth rate of cells (μ) and on the intracellular protein content, the specific production rate of proteins was evaluated. Thus, based on the occurrence of each amino acid in proteins, their respective specific rates of use required to

synthesize the proteins are found (n=31+20=51). In the calculation of these rates we have taken into account the recycling of amino acids occurring during the destruction and rebuilding of proteins.

Table 4-3: Composition of nucleic acids in lepidopteran cells

Composition of DNA in lepidopteran cells

Base	Occurrence (%)[1]
A	0.32
T	0.32
G	0.179
C	0.179

Estimated composition of purines in lepidopteran cells

Compounds	Required amount to synthesize one purine[2]
ribose	1
phosphate	1
Gln	1.79
Gly	0.32
Asp	1.32
NH3	0.25

Estimated composition of pyrimidines in lepidopteran cells

Compounds	Required amount to synthesize one pyridine[2]
ribose	1
phosphate	1
Gln	2.11
Gly	0.50
Asp	1.32
NH3	0.25

[1] percentages in molar, calculated on the basis of A/T, G C and A+T/G C molar ratios of *Bombyx mori* from Sober et al. (1970)
[2] calculated from the quantity of each compound required to synthesize one base and normalized according to the occurrence of that same compound in each base.

Concerning DNA (Figure 4-3) and RNA (data not shown), a similar approach was taken. The occurrence of each base was evaluated based on the data of Sober et al. (1970) for the lepidopteran species *Bombyx mori* (data found to be the closest match to *Trichoplusia ni* and *Spodoptera frugiperda*). Based on the Asp, Gly, Gln, NH_3, ribose and phosphate required to synthesize each base, on the growth rate and on the average MW of one base (76.5 g/mol for one purine and 79.9 g/mol for one pyrimidine), the specific rates of

consumption of Asp, Gly, Gln, NH_3 and Ribose-5-P used to synthesize DNA and RNA were found (Table 4-3) (*n*=51+5=56).

As for carbohydrates, they are mainly present in the glycoproteins of cellular membranes (Davis et al. 1993). Although the types of sugars present in insect cell glycoproteins change as a function of culture conditions and cell line (Palomares et al. 2004), variations represent only micro-heterogeneity and, generally, the "average" glycosylation is stable (Hollister et al. 2002; Palomares et al. 2004). This "average" glycosylation (Man7GlcNAc2) contains from 5 to 9 mannose and 2 N-acetylglucosamine groups (with an average of 9 sugars of 6.25 carbons, *i.e.* with 171.1 g/mol of MW for each). With the same methodology as for proteins, the specific rate of consumption of sugars to produce carbohydrates is found (*n*=56+1=57).

Regarding lipids, their occurrence in cells was extrapolated from phospholipids and glycolipids in mosquito cells (the nearest data found, Luukkonen et al. 1973) and from data on triglycerides, free fatty acids and sterols occurring in High-Five and Sf-9 Cells (Marheineke et al. 1998) (Table 4-4). The "average" lipid in insect cells contains 34.1 C atoms in hydrocarbonated chains (MW of one lipid: 826 g/mol).

Table 4-4: Lipid composition in insect cells

Lipid composition in insect cell

Lipids	Total lipid occurrence (%) [1]	Occurrence in High-Five (%) [2]	Occurrence in Sf-9 (%) [2]
Phospholipids	81.0 ± 2.6	-	-
Glycolipids	0.5	-	-
Triglycerides	10.7	78	72
Free fatty acid	1.8	5	5
Sterols and chlolesterols	3.1	17	23
Esters sterols	2.8		

Estimated lipid composition in High-Five and Sf-9 cells

Quantity of lipids containing one glycerol[3]	91 %
Lenght of hydrocarbonated chains[4]	17.6 C atoms
Numbers of hydrocarbonated chain per lipid[3]	1.94

[1] total lipid occurrence (percentages in w/w) in the insect *Aedes albopictus* calculated from Luukkonen et al. (1973)
[2] occurrence of lipids except phospholipids and glycolipids (percentages in w/w) in *Spodoptera frugiperda* and *Trichoplusia ni* cells calculated form Marheineke et al. (1998)
[3] calculated form the weighted number of glycerol and hydrocarbon chains in each type of lipid and the occurrence of each type of lipid in insect cells from Luukkonen et al. (1973) and Marheineke et al. (1998)
[4] calculated from the average length of hydrocarbon chain from Marheineke et al. (1998)

These occurrences, the number of glycerol moieties and number of carbon atoms in the hydrocarbon chain necessary to synthesize each type of lipid weighted by the proportion of each lipid in insect cells (Table 4-4) and the growth rate were used to estimate the specific rate of use of glycerol-3-P and palmitate (giving 2 carbon atoms to lipids during elongation) to synthesize the lipids (n=57+2=59).

Therefore, the total known (n) fluxes is 59 and the number of unknown fluxes is 87 (p-n). A single piece of data (n) is missing to solve the system: $\boldsymbol{p - m} = 146 - 86 = 60 > \boldsymbol{n} = 59$. In order to solve the system and to make it applicable for each set of conditions, the amino acid with the lowest difference between its extracellular specific consumption rate and its intracellular specific rate of biomass synthesis (sum of its fluxes leading to protein, DNA and RNA synthesis) was assumed to be only used in anabolic pathways (often: Leu, Lys, Ile, Phe, Tyr or Val). Hence, its catabolic flux was set equal to *zero* (n= 59+1=60). The system, set up in matrix form (Equation 4-1), was solved with Mathematica 4.0 using the "Solve[D.E==F] //N" function (Wolfram Research, Oxfordshire, UK). Each one of the reactions producing or consuming ATP, NADH, $FADH_2$, O_2 and CO_2 was identified (Figure 4-1) and used to estimate the specific production rate of ATP, O_2 and CO_2 (shown in Tables 4-5, 4-6 and 4-7 and in Figure 4-3). In order to control the accuracy of the metabolic network, for each metabolite, we have simulated a consumption/production rate of 1 mol/10^9cells.s of this metabolite with rates of the other metabolites set up to zero, to check if the resolution of the equation system gave coherent values. For each condition, once the equation system was solved, we have checked that all anabolic fluxes were strictly positive. Due to the great number of data obtained from the resolution of the metabolic flux network under various culture conditions, only selected and summarized data are presented in the remaining Figures and Tables.

4.3.2. Analysis of the metabolism of uninfected High-Five cells

We used the network presented in Figure 4-1 to study the metabolism of High-Five and Sf-9 cells during their exponential growth phase and during their infection phase. The **m**easurement of **s**pecific consumption/production **r**ates (MSR) of **e**xtracellular **f**luxes (EF), of High-Five cells during their growth phase, showed (Figure 4-2) that these cells, cultivated in YPR, consumed high amounts of glucose, but rather low amounts of amino acids (except Gln and Asn) and produced high levels of lactate, Ala and ammonia (Table 4-5 and Figure 4-2).

Figure 4-2 (next page): Metabolism of High-Five cells
The thickness of the lines represents the magnitude of the flux as in the framed legend.
Lipids, FA, FA carnitine and FA-CoA are expressed in terms of µmol of C atom in hydrocarbonated chain(s) / 10^9 cells.h

malt
gluc
sucr
fruct
cystine
Trp
Ser
Gly
succ
fum
α-keto
mal
lac
ethanol
CO_2
O_2
FA
FA car
FA-CoA
Ala
Ile
Leu
Lys
His
Pro
Glu
Arg
Gln
PROTEINS
nucleus
urea
NH_3
Glu α-keto
prop-CoA
acetoacet
acetyl-CoA
cit
isocit
α-keto
succ-CoA
succ
fum
oxalo
mal
pyr
Cys
Ser
Met
Thr
Val
Phe
Tyr
Asn
Asp
Gly
Mitochondrion
CARBOHYDRATE
rib5P
G6P
FBP
G3P
gly3P
LIPIDS
palmitate
mal-CoA
acetyl-CoA
Nucleus
PUR / PYR
DNA / RNA
Gly
Gln
Asp
NH_3
CO_2
Cytosol
Extra-cellular
Theshold :
µmol / 10^9 cells . h
> 200
100-200
50-100
20-50
High-Five cells
in growth phase
10-20
5-10
2-5
0.5-2
< 0.5

The quantitative assessment of **i**ntracellular **f**luxes (IF) based on the **a**nalysis of the **m**etabolic **f**lux **n**etwork (AMFN) indicated that glucose was used principally to feed the Krebs cycle, the lactic fermentation and the production of Ala. Few sugars were used to synthesize lipids, ribonucleotides and carbohydrates (Figure 4-2). The amino acids were mainly consumed to synthesize proteins (as seen from AMFN, Figure 4-2), except for Ala that was a by-product.

Table 4-5: Effect of different media compositions on the metabolite consumption/production by High-Five uninfected cells during exponential phase (between 24 and 96 h or as specified)

		High Glucose	Mean Glucose	Low Glucose	High Asn [1]	High Gln [1]	With exogenous 5 mM lactate [2]	With 5 mM glucose and 10 mM lactate [2]
Media composition at the inoculation (mM)	Glucose	**55**	34	12	55	55	55	5
	Lactate	**0**	0	0	0	0	5	10
	Asn	**12**	12	12	25	12	12	12
	Gln	**12**	12	12	12	25	12	12
Specific rate of consumption/production (µmol / 10^9 cells.h)	Glucose	**123.9**	164.7	185.6	149.0	154.0	224.3	6.6
	Lactate	**5.8**	19.0	40.2	21.6	48.2	29.4	52.6
	NH_3	**55.2**	81.6	66.0	112.6	139.5	110.7	104.2
	Ala	**32.5**	19.7	14.7	63.3	115.0	25.8	**-62.2**
	Gln	**34.6**	48.2	38.7	17.2	20.8	35.2	20.9
	Glu	**14.8**	2.0	1.1	12.0	16.5	5.6	23.2
	Asn	**30.2**	33.5	9.1	84.9	40.0	49.3	37.4
	Asp	**3.3**	1.2	3.1	9.7	19.0	8.9	18.7
Energy : ATP production (% of the total ATP produced)	Krebs Cycle	**60.6**	66.9	59.7	66.0	56.7	71.7	7.1
	Glycolysis	**16.2**	17.8	16.0	17.6	15.1	19.1	1.9
	Lactic fermentation	**0.2**	0.1	0.4	3.0	0.6	0.3	0.7
	Asn+Asp consumption	**3.7**	6.6	0.5	7.3	15.4	4.0	30.2
	Gln+Glu consumption	**3.4**	3.8	1.4	1.9	8.7	0.8	11.9
	Lipid consumption	**4.1**	4.0	4.8	3.4	3.0	3.6	0.7
	Other pathway	**11.8**	0.8	17.2	0.5	0.4	0.5	47.6
Nitrogen production (% of the total N consumed)	Gln consumption	**8.6**	6.8	19.2	11.0	17.2	28.2	1.8
	Glu consumption	**3.8**	2.3	1.2	9.7	3.0	6.3	1.6
	Asn consumption	**46.0**	34.5	6.3	68.0	21.3	55.2	1.5
	Asp consumption	**3.2**	1.9	1.2	4.2	4.3	3.4	1.5
	Other pathway	**38.4**	54.5	72.1	7.1	54.2	6.9	93.6
Ratio NH_3 + urea / Ala (specific rate of production)		**1.82**	4.08	4.23	1.74	1.23	4.25	-1.68

[1] The data of culture conditions in high Asn and high Gln are average values taken during exponential phase (between 24 and 72 h).
[2] The data of cultivation with exogenous lactate and low glucose are average values taken during exponential phase (between 0 and 48 h).
Note : the medium with 55 mM of glucose and 12 mM of Asn and 12 mM Gln is the standard YPR medium

Asn and Gln are used to produce energy *via* anaplerotic pathways that convert Asp to oxaloacetate and Glu to α-ketoglutarate (AMFN). Among the other amino acids, the catabolic fluxes were low for Arg, Asp, Cys, Glu, His, Lys, Met, Thr ; very low for Ile, Ser, Tyr, and almost insignificant for Gly, Leu, Phe, Pro, Trp, Val. Malate was the only organic acid present in the Krebs cycle that had a significant rate of consumption (AMFN, Figure 4-2). The production of urea and ethanol as by-products proved almost insignificant.

The consumption rate of fatty acids to produce energy or lipids was low (AMFN, Figure 4-2) and the production of lipids required the use of the citrate produced by the Krebs cycle. Most of the ATP came from the Krebs cycle (~60 %) and glycolysis (~16 %) (AMFN, Table 4-5). The consumption of Asn/Asp and Gln/Glu accounts for 8.1 % of the ATP produced (AMFN, Table 4-5), whereas the lactic fermentation does not produce energy.

The consumption of oxygen and production of CO_2 expected from the metabolic flux model (AMFN: 655 µmol O_2 and 700 µmol $CO_2/10^9$cells.h, Table 4-7) was confirmed by the experimental data (MSR: 620 ± 35 µmol O_2 and 640 ± 75 µmol $CO_2/10^9$cells.h, data not shown).

Various observations emerged when we compared cells cultivated in high glucose medium to low glucose (Table 4-5). The consumption rate of glucose decreased with the increase of the initial glucose concentration in the medium (same initial amino acids concentration). However, the cells wasted the energy available in the increased glucose level by producing higher amounts of by-products (MSR). In high glucose medium, the cells used more Gln than Asn, as energy source and produced more ammonia than Ala.

Table 4-6: Comparison of the catabolism of infected (0 to 96 hpi) and uninfected (24 to 96h) High-Five and Sf-9 cells cultivated in YPR medium

		Growth phase		Infection phase	
		High-Five	Sf-9	High-Five	Sf-9
Specific rate of consumption (µmol / 10^9 cells.h)	Glucose	123.9	93.5	164.1	59.8
	Lactate	5.8	4.0	13.7	2.7
	Gln	34.6	23.3	66.6	19.2
	Glu	14.8	10.3	13.7	5.5
	Asn	30.2	9.0	32.2	3.5
	Asp	3.3	5.1	14.9	3.5
Specific rate of production (µmol / 10^9 cells.h)	NH_3	55.2	17.9	38.6	22.4
	Ala	32.5	41.4	46.0	27.4
Ernegy : ATP production (% of the total ATP produced)	Krebs Cycle	60.6	59.3	69.2	73.3
	Glycolysis	16.2	15.8	18.4	19.5
	Lactic fermentation	0.2	0.2	0.2	0.1
	Asn+Asp consumption	3.7	4.3	0.8	0.2
	Gln+Glu consumption	3.4	16.7	6.6	3.9
	Lipid consumption	4.1	1.9	0.8	0.5
	Other pathway	11.8	1.9	4.0	2.5
Nitrogen production (% of the total N consumed)	Gln+Glu consumption	12.2	63.5	59.4	61.8
	Asn+Asp consumption	49.2	20.2	30.1	11.9
	Other pathway	38.6	16.3	10.5	26.3
Ratio NH_3 + urea / Ala (specific rate of production)		1.82	0.53	0.92	0.83

Table 4-7: Comparison of the anabolism of infected (0 to 96 hpi) and uninfected High-Five and Sf-9 cells cultivated in YPR medium

		Growth phase		Infection phase	
		High-Five	**Sf-9**	**High-Five**	**Sf-9**
Specific rate of consumption (µmol / 10^9 cells.h)	Total carbohydrates [1]	123.9	93.5	164.1	59.8
	Total Gln-Glu-Asn-Asp	72.9	47.7	127.4	31.7
	Total free amino acids [2]	116.4	130.0	153.0	88.0
	Total amino acids [3]	155.9	144.6	171.4	94.2
	Total lipids (µmol of C atom / 10^9 cells.h) [5]	4.8	4.0	3.5	> 1
Specific rate of production (µmol / 10^9 cells.h)	Lactate and ethanol by-products	5.9	9.0	13.8	2.7
	NH_3, Ala and urea by-products	92.8	60.2	85.6	50.1
Specific rate of consumption	O_2	655	460	1229	486
Specific rate of production (µmol / 10^9 cells.h)	CO_2	700	509	1273	499
	ATP	4139	2915	7779	3060
Use of ATP [4] (%)	ATP used for growth: cellular biomass synthesis	73	76	16	14
	ATP used for maintenance	27	24	15	23
	ATP used for infection: r-protein(s) and virus synthesis	-	-	69	63
Specific rate of synthesis	Cellular and/or viral proteins (mg / 10^9 cells. h)	12.2	6.3	5.0	1.3
	Extracellular r-proteins (mg / 10^9 cells. h)	-	-	9.5	7.3
	Cellular and/or viral DNA (µg / 10^9 cells. h)	31.3	17.7	8.3	1.7
	Cellular and/or viral RNA (µg / 10^9 cells. h)	128	67	45	5.6
	Carbohydrates (µg / 10^9 cells. h)	269	179	100	9.1
	Cellular and/or viral lipids (µg / 10^9 cells. h)	797	624	208	44
Specific rate of synthesis	Cellular and/or viral proteins (µmol amino acid/ 10^9 cells. h)	109.7	56.8	44.8	11.4
	Extracellular r-proteins (µmol amino acid / 10^9 cells. h)	-	-	85.4	65.7
	Cellular and/or viral DNA (µmol base / 10^9 cells. h)	0.41	0.23	0.11	0.02
	Cellular and/or viral RNA (µmol base / 10^9 cells. h)	1.60	0.84	0.56	0.07
	Carbohydrates (µmol carbohydrate / 10^9 cells. h)	1.57	1.05	0.58	0.05
	Lipids (µmol of C atom/ 10^9 cells.h) [5]	37	26	8.6	1.80

[1] glucose, maltose, fructose and sucrose.
[2] except for Ala that is produced and not consumed
[3] free amino acids plus amino acids present in hydrolysates
[4] calculation based on the growth rate, on the biomass composition of cells and on the production rate of ATP during stationary phase (only maintenance), exponential phase (growth + maintenance) and infection phase (infection +maintenance)
[5] µmol of carbon atom equivalents in the hydrocarbonated chains of lipids /10^9 cells.h

Table 4-8: Use of ^{14}C radiolabelled asparagine by insect cells cultivated in different media

		Sf-9	High-Five			Sf-9	High-Five		
Media composition	Glucose (mM)	55	**55**	12	12	55	**55**	12	12
	Asn (mM)	12	**12**	12	25	12	**12**	12	25
Fate of the radiolabelled asparagine consumed		(% of ^{14}C atom produced)[1]				(% of radiolabelled compounds)[2]			
Production of lactate		0.7	**0.3**	1.1	1.9	1.0	**0.3**	1.4	2.5
Production of Ala		2.8	**1.2**	3.8	8.9	3.7	**1.7**	5.1	11.9
Production of Asp		3.9	**7.8**	17.7	18.5	3.9	**7.8**	17.7	18.5
Production of CO_2 [3]		68.9	**68.5**	51.3	44.5	275	**274**	205	177
Accumulation of cell biomass		8.5	**15.2**	15.8	18.0	-	-	-	-
Other pathways [4]		15.2	**7.0**	10.3	8.2	-	-	-	-

The flux values were determined from radioactive experiments as described in Materials and Methods.
[1] based on the cell densities, on the consumption rates of Asn^{14}C (dpm/vial.day) and on the production rates of ^{14}C compounds (dpm/vial.day) after separation by TLC
[2] based on the ^{14}C produced as %, on the number of C atoms present in each compound and on the MW of each compound (Ala: 3C, Asn: 4C, Asp: 4C, CO_2: 1C, lactate: 3C). Note: their sum could be higher than 100 %
[3] estimated from the $^{14}CO_2$ trapped into the hyamine solution
[4] deduced by the difference between the total consumption of Asn^{14}C and the total of the production of lactate^{14}C, Ala^{14}C, Asp^{14}C, $^{14}CO_2$ and the accumulation of Asn^{14}C in biomass
Note : the medium with 55 mM of glucose and 12 mM of Asn is the standard YPR medium

When the High-Five cells were cultivated in the presence of exogenous lactate, the production of the toxic by-products lactate and ammonia increased (MSR, Table 4-5). When the cells were at the end of the growth phase (near the onset of the stationary phase), the glucose concentration became low, while the lactate and ammonia concentrations were high (MSR). In these conditions, cells consumed less glucose to produce ATP and used the lactic acid as energy source (AMFN, Table 4-5). The cells also consumed the Ala and produced very high amounts of ammonia (MSR).

In the presence of high Gln or Asn concentration, the cells increased considerably their respective Gln or Asn consumption, and the production of ATP by anaplerotic pathways increased. The contribution of amino acids to the production of ammonia and Ala increased (AMFN, Table 4-5 and Figure 4-2).

A kinetic study showed that, in absolute terms, the specific rate of glucose consumption decreased during the cultivation in YPR medium. The consumption of glucose by High-Five cells decreased from 143 µmol/10^9cells.h during the 24 first hours (exponential phase) to124 µmol/10^9cells.h in mid-exponential phase (at 48 and 72 h), to 104 µmol/10^9cells.h at the end of the growth phase (at 120h) and down to 85 µmol/10^9cells.h during stationary phase (at 144 h), the latter values being associated to a drastic decrease of the growth rate when the glucose concentration decreased.

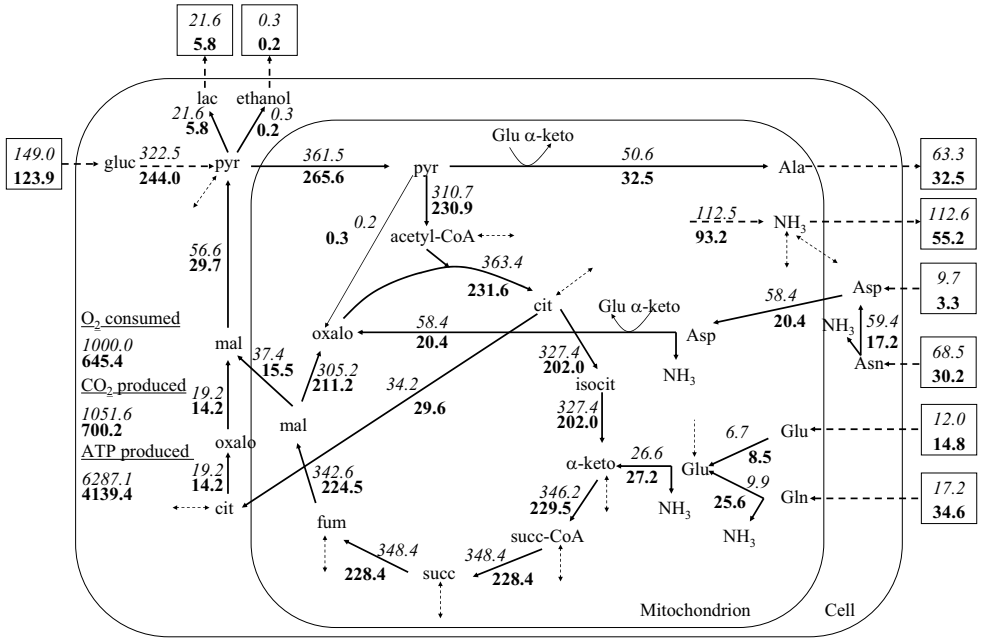
The particularity of the consumption of Asn by High-Five cells in the metabolic network (Figure 4-3) was studied more closely using Asn^{14}C (Table 4-8). The goal of this

experiment was to obtain an experimental confirmation of the fluxes found in our metabolic flux network. We have previously shown that High-Five cells cultivated in YPR medium consumed Asn at a higher rate than Gln (Tables 4-5, 4-6 and 4-7). The respective fluxes of the central catabolism of High-Five cultivated in YPR medium (12 mM Asn) and in high Asn medium (25 mM Asn) are presented in Figure 4-3 (AMFN). The data represent average fluxes (μmol/10^9cells.h) during the first 72 hours of cultivation, when Asn was not completely consumed. In standard YPR medium (glucose 55 mM, Gln 12 mM and Asn 12 mM), during the lag phase, the cells consumed more Asn, and less glucose and Gln, than in exponential phase. Cultivated in high Asn medium, the specific consumption rate of this amino acid was very high and cells had the tendency to consume almost all Asn in the first days of cultivation, before starting to consume high amount of Gln (MSR, data not shown).

Cultivated in high Asn medium, the cellular fluxes of conversion of malate to pyruvate doubled (AMFN), the production of Ala and ammonia also doubled and the production of lactate was 4 times higher (MSR), whereas the fluxes of consumption of glucose were slightly increased and the consumption of Gln was 5 times lower (MSR, Figure 4-3) compared to standard YPR medium. In high Asn medium (25 mM Asn) and in high Gln medium (25 mM Gln), High-Five cells consumed more glucose, and produced more lactate than in standard YPR medium (MSR, Table 4-5). The majority of nitrogen by-products produced came from Asn except in high Gln medium (Table 4-5 and Figure 4-3). The specific production rate of ATP was higher when cells were cultivated in high Asn medium (AMFN). Nevertheless, the growth rate was not augmented.

The experiment of the addition Asn^{14}C in the medium (Table 4-8) showed that cells consuming Asn^{14}C produced ^{14}C labelled Ala, Asp, lactate and CO_2. We did not find any radiolabelled ethanol and glucose. According to the results of this experiment, when High-Five cells were fed with Asn^{14}C, they produced more ^{14}C lactate and Ala than Sf-9 cells (Table 4-8). When cultivated in YPR medium, they consumed 15.2 % of the Asn^{14}C for biomass production, by using directly Asn (for protein synthesis) or after converting it to Asp (for protein and ribonucleotide synthesis) whereas our flux network analysis indicated that 13.0 % of Asn is used for anabolism (data not shown). In these conditions, the cells produced high $^{14}CO_2$ (68.5 %) from Asn^{14}C (Table 4-8) which indicated a high use of ^{14}CAsn to feed the Krebs cycle (see Discussion). When the High-Five cells were cultivated in YPR medium with high Asn concentration (25 mM), they consumed more Asn^{14}C and produced more Ala^{14}C and lactate^{14}C than in low Asn medium (12 mM).

Figure 4-3: Central metabolism of High-Five cells during cultivation under standard conditions (12 mM Asn) and with high Asn concentration (25 mM).
The lower numbers in bold represent the values of the fluxes (in µmol / 10^9cells.h) when High-Five cells were cultivated in standard YPR medium. The upper numbers in italics are the fluxes when cells were cultivated in YPR containing 25 mM Asn. Full lines correspond to a single and unique typical metabolic pathway, dashed lines represent interactions with other portions of central metabolism (not shown here).



4.3.3. Analysis of the metabolism of uninfected Sf-9 cells

The metabolism of lepidopteran cell lines is known to be well conserved in evolution. The Sf-9 and High-Five cells have qualitatively the same metabolic pathways, but different flows. Overall, Sf-9 cells have lower specific rates of consumption/production than High-Five cells (Figures 4-2 and 4-5), although they generally maintain the same proportions in these fluxes. Yet, Sf-9 cells are physically smaller than High-Five and have a lower cellular biomass content (Table 4-1). The anabolic pathways for Sf-9 biomass production (proteins, lipids, ribonucleotides and carbohydrates) were found to be similar to those of High-Five cells (AMFN, Figure 4-5). In spite of these similarities, several differences between the two cell lines were observed.

Sf-9 cells consumed less glucose, Asn, Gln and carbohydrates per cells than High-Five cells. They consumed lipids at an almost equal rate to High-Five cells (MSR, Tables 4-6 and 4-7). On the other hand, Sf-9 cells had a lower consumption of amino acids from

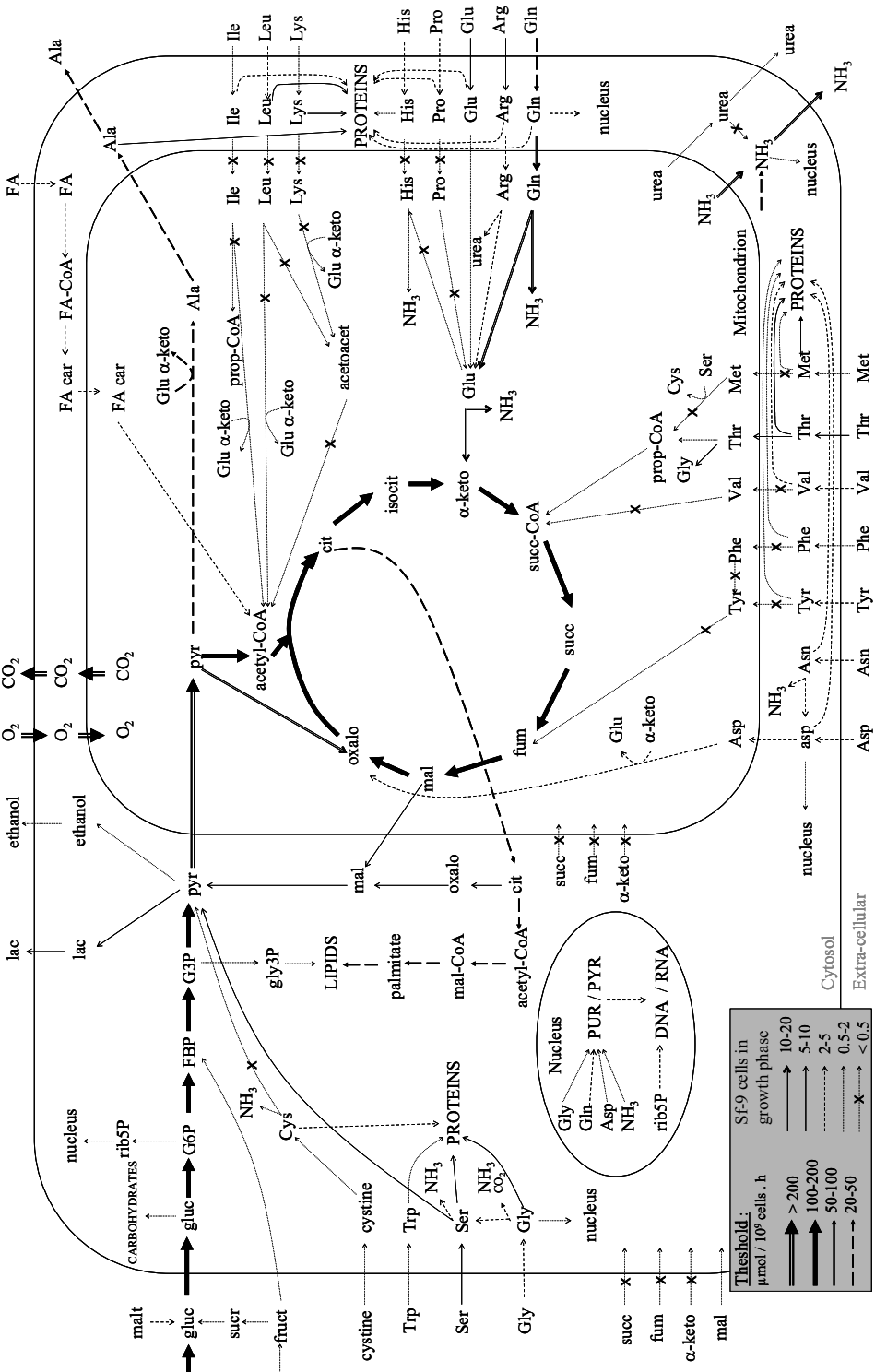
peptones (Yeastolate and Primatone) than High-Five cells (MSR, Table 4-7). Sf-9 cells used the bulk of the amino acids (87%) to synthesise proteins except for Asn, Asp, Gln and Glu for which 48 % (Asn), 98 % (Asp), 27 % (Gln) and 43 % (Glu) respectively, were used for catabolism (AMFN, Figure 4-5). Gln appeared to be a high energy source (accounting for 16.7 % of the ATP produced), and its consumption was the major anaplerotic pathway of Sf-9 cells, as opposed to Asn for High-Five cells (AMFN, Table 4-6). Contrary to High-Five, Sf-9 did not significantly use His, Ilea, Met and Tyr in catabolic pathways (AMFN, Figure 4-5).

Concerning by-products, Sf-9 cells produced proportionally more Ala, less ammonia and less lactate than High-Five cells (MSR). In central catabolism, the export of citrate from the mitochondrion to the cytosol (AMFN) was relatively constant for both cell lines (23.4 µmol/10^9 cells.h for Sf-9 cells, data not shown; 29.6 µmol/10^9 cells.h for High-Five cells, Figure 4-3). However, the transformation of malate to pyruvate in the cytosol was lower in Sf-9 cells (8 µmol/10^9cells.h, data not shown, vs. 29.7 for High-Five, Figure 4-3).

4.3.4. Analysis of the metabolism of infected cell lines

During infection, various changes occurred. High-Five cells displayed increased consumption of glucose, contrary to Sf-9 cells (Table 4-6). Infected High-Five cells produced more lactate, less ammonia and less Ala compared to uninfected High-Five cells (MSR). Infected Sf-9 cells produced less lactate and "nitrogen by-products" than uninfected Sf-9 cells (MSR). During infection, the ratio NH_3 + urea / Ala tended towards unity for both cell lines, whereas uninfected High-Five and Sf-9 cells had a respective ratio of 1.82 and 0.53 (MSR). The consumption of Gln by High-Five increased, while that of Asn was similar to the one during the exponential phase. The consumptions of Asn, Asp, Gln and Glu by infected Sf-9 decreased during infection (MSR). As for the other amino acids, their consumption during infection, by both cell lines, served essentially for synthesis of proteins and ribonucleotides during infection (AMFN, data not shown).

Figure 4-5 (next page): Metabolism of Sf-9 cells
The thickness of the lines represents the magnitude of the flux as in the framed legend
Lipids, FA, FA carnitine and FA-CoA are expressed in terms of µmol of C atom in hydrocarbonated chain(s) / 10^9 cells.h


Ala
FA
FA car
FA-CoA
Ile
Leu
Lys
His
Pro
Glu
Arg
Gln
PROTEINS
nucleus
urea
NH3
Glu α-keto
prop-CoA
acetoacet
Mitochondrion
Cys
Ser
Gly
Met
Thr
Val
Phe
Tyr
Asn
Asp
asp
cit
isocit
α-keto
succ-CoA
succ
fum
mal
oxalo
acetyl-CoA
pyr
CO2
O2
ethanol
lac
gly3P
LIPIDS
palmitate
mal-CoA
Nucleus
PUR / PYR
DNA / RNA
Gly
Gln
Asp
NH3
rib5P
CARBOHYDRATES
malt
gluc
sucr
fruct
G6P
FBP
G3P
cystine
Trp
Ser
Cytosol
Extra-cellular
Theshold :
µmol / 10⁹ cells . h
> 200
100-200
50-100
20-50
Sf-9 cells in
growth phase
10-20
5-10
2-5
0.5-2
< 0.5

For both cell lines, during infection, most of the ATP was used for virus particle replication and recombinant protein (β-gal) production (AMFN). The latter represents 69 % and 63 % of the ATP used during the infection for High-Five for Sf-9 (AMFN, Table 4-7). The overall rates of protein synthesis were the same as in uninfected cells but the proteins synthesized were principally recombinant or viral. During infection, the production of cellular DNA and RNA decreased by four for High-Five cells, and by ten for Sf9 cells (AMFN). The total arrest of the growth indicated that the virally encoded proteins and ribonucleotides were only synthesized during the infection process. For both cell lines, the synthesis of cellular carbohydrates and lipids decreased during the infection phase (AMFN, Table 4-7).

4.4. DISCUSSION

4.4.1. METABOLISM OF UNINFECTED HIGH-FIVE AND SF-9 CELLS

The analysis of the catabolism and the anabolism of High-Five and Sf-9 cells, in different cultivation environments, first and foremost tend to highlight differences between these two cell lines and mammalian cells. Insect cells have higher specific consumption of glucose and oxygen than mammalian cells, while the production of lactate is lower (Neermann and Wagner 1996) and the consumption of amino acids, lipids and ammonia are higher. Furthermore, there are several key differences between Sf-9 and High-Five insect cell lines. This work confirms and extends the findings of Rhiel et al. (1997) and Benslimane et al. (2005).

4.4.1.1. Catabolism

Carbohydrate consumption

We showed that both High-Five and Sf-9 cells use preferentially glucose (q_s ~ 150 µmol/10^9cells.h) (MSR, Figures 4-2 and 4-5, Tables 4-5 and 4-6). As reported by Öhman et al. (1995) and Rhiel et al. (1997), Sf-9 cells are also able to consume other carbohydrates but at a very low rate upon cultivation in rich media. High-Five cells have higher specific metabolism rate for glucose in their growth phase than Sf-9. Contrary to Benslimane et al. (2005) who have recently reported that the main mode of glucose utilization by Sf-9 and High-Five cells cultivated in SF900-II and Excel 405 made use of the pentose phosphate pathways, our own metabolic network (Figures 4-2 and 4-5, Tables 4-5, 4-6 and 4-7), and our measurements of oxygen consumption and CO_2 production (MSR) show that glucose is mainly used by High-Five and Sf-9 cells cultivated

in YPR medium to feed the Krebs cycle rather than for lactate and/or Ala production. An obvious proof of this point is that High-Five cells cultivated in YPR produce 5.6 moles of CO_2 per mole of glucose consumed and consume 5.2 moles O_2 / mole glucose (AMFN and MSR, Table 4-7).

Lipid consumption

Both High-Five and Sf-9 cells use lipids to a limited extent as energy source (AMFN, Table 4-6, Figures 4-2 and 4-5). Their consumption by the cells is lower than their biosynthesis from citrate (Table 4-7). Thus, the addition of lipids in the medium is only required because lepidopteran cells need some sterols (5.9 % of their total lipids) that they cannot synthesize on their own (Goodwin 1991).

Energy production

Concerning the production ATP, the complete oxidation of one mole of glucose produces 38 moles of ATP by glycolysis and the Krebs cycle, whereas 12 moles of ATP are produced from Asn and 21 from Gln as an energy source in higher eukaryotic cells (Murray et al. 1996). However, if the oxidation is incomplete, the production of one mole of lactate wastes 18 moles of ATP, and 15 moles of ATP for one mole of Ala produced (Murray et al. 1996). Based on these considerations and on the specific production rate of Ala, lactate (MSR, Table 4-6) and ATP (AMFN, Table 4-7), High-Five and Sf-9 cells appear to waste respectively 14 and 24 % of the available energy present in their substrates, due to the production of Ala and lactate instead of the total oxidation of glucose, Gln and Asn in the Krebs cycle (Mendonça et al. 1999).

By-product accumulation

Lactate production

With respect to metabolic by-products, insect cells commonly display lower specific production rate of lactate (MSR, Table 4-6) than mammalian cells (*e.g.* CHO cells). Insect cells have a higher consumption of amino acids as energy source which leads to a higher production of ammonia and Ala (MSR, Table 4-6) than mammalian cells. The lower formation of lactate is explained by the fact that insect cells are using the major part of the pyruvate produced to feed the Krebs cycle. This production of lactate is more intense in High-Five than Sf-9 cells (MSR, Table 4-6 and Bédard et al. 1993), but remains lower than in mammalian cells, except if the insect cells are cultivated in sub-optimal medium *e.g.* High-Five cells in Sf900-II (Ikonomou et al. 2003). Sf-9 cells usually produce very little lactate in a non-limiting oxygen environment (MSR, Table 4-6 and Ikonomou

et al. 2003). High-Five cells were shown to produce more lactate when exogenous lactate was present in the culture (Table 4-5) and tended to waste the available energy present in glucose (Drugmand et al. 2005). This production of lactate increases when High-Five cells are stressed e.g. by the infection process (MSR, Table 4-6), the lack of glucose (MSR, Table 4-5), etc. while its production tends to stress the cells even more and to further increase lactate production causing a "snowball" effect, as already described by Drugmand et al. (2005).

Ammonia production

High-Five cells produce more "nitrogen by-products" (ammonia and its detoxified form: Ala) than Sf-9. However, Sf-9 cells produce more Ala than High-Five (MSR, Table 4-6). This accumulation in the medium depends on the initial concentration of Asn, Asp, Gln and Glu (Ikonomou et al. 2003). This formation of Ala as by-product wastes some available energy, but prevents the production of ammonia, to which Sf-9 cells are known to be more sensitive than High-Five and this could explain why Sf-9 cells tend to produce less ammonia than High-Five (Bédard et al. 1993).

Alanine production

When High-Five cells are cultivated in conditions of low glucose and high lactate (as during the stationary phase), they are able to use the lactate and Ala accumulated during the exponential phase to produce energy (Table 4-5, Drugmand et al. 2005). The consumption of lactate provides little energy to the cells and most ATP produced in the stationary phase comes from the Ala (AMFN, Table 4-5). Upon glucose exhaustion, ammonia begins to accumulate and Ala concentration drops in Sf-9 insect cell culture (Bhatia et al. 1997). Both Sf-9 and High-Five cells are able to consume the Ala as energy source (AMFN, Figures 4-2 and 4-5, Tables 4-5 and 4-6) producing pyruvate in the mitochondrion by the reversible alanine aminotransferase enzyme (also called glutamate:pyruvate transaminase) (Bédard et al. 1993) and to channel this pyruvate into the Krebs cycle to produce ATP (Figures 4-1 and 4-6: enzyme 6, Reaction 4-1).

$$\text{Ala} + \alpha\text{-ketoglutarate} \leftrightarrow \text{pyruvate} + \text{Glu} \qquad \text{(Reaction 4-1)}$$

The Glu produced is converted to α-ketoglutarate by producing NH_3 by glutamate dehydrogenase (Drews et al. 2000; Öhman et al. 1996) (Figures 4-1 and 4-6: enzyme 8, Reaction 4-2).

$$\text{Glu} + NADP^+ \rightarrow \alpha\text{-ketoglutarate} + NADPH + NH_3 \qquad \text{(Reaction 4-2)}$$

Waste of nutrients

The more glucose the insect cells consume, the more available energy they waste (MSR, High-Five cells: Table 4-5, Sf-9 cells: data not shown). The lactate consumed by High-Five cells does not produce ATP by the lactic fermentation but rather produces pyruvate that can enter into the Krebs cycle to produce ATP (7.1 % of the ATP produced by High-Five cells cultivated into low glucose and high lactate conditions, AMFN, Table 4-5). In these conditions, High-Five cells consume also more Asp and Gln and less glucose than in standard, less stressful conditions. On the other hand, we have not observed the consumption of the accumulated lactate and Ala by-products by Sf-9 cells (data not shown, Ikonomou et al. 2001) probably because Sf-9 cells become more rapidly pre-apoptotic at the end of the growth phase or because of defective enzymes.

Other by-products

Concerning the minor by-products (urea and ethanol), we observed that their production by insect cells (MSR, High-Five: Figure 4-2, Sf-9: Figure 4-4) is minimal (below 1 % of the overall production of metabolic by-products) as previously shown by Wyatt (1956) and Takahashi et al. (1995). Urea is only formed when the insect cells consume Arg to produce energy while ethanol is produced as an alternative pathway to the lactic fermentation. The toxicity of these minor metabolites is not consequential due to the low levels produced.

Amino acid consumption

We have shown in this work that the majority of the amino acids consumed contribute to the production of energy in the form of ATP and the synthesis of proteins and ribonucleotides (AMFN, Figures 4-2 and 4-4). The hydrolysis of peptides seems to provide only little energy to the insect cells (Table 4-7, Vaughn and Fan 1997). Generally, Sf-9 cells use amino acids to a lesser extent than High-Five cells for catabolism (AMFN, Figures 4-1 and 4-2, Table 4-7). In addition of glucose, Sf-9 cells use almost exclusively Gln as energy source, whereas High-Five consume Asn and Gln to produce energy (AMFN, Table 4-6). We have documented a consumption of every amino acid present in the metabolic network (Figure 4-2). Among all these amino acids, Arg, Gly, His, Ile, Leu, Lys, Met, Phe, Pro Thr, Trp, Tyr and Val are essential, i.e. required amino acids to sustain the growth of lepidopteran cells lines cultivated *in vitro* according to the studies by Bhatia et al. (1997), Bédard et al. (1993), Ferrance et al. (1993), Landureau (1969), Landureau and Jollès (1969) and Radford et al. (1997). Drews et al. (1995) reported that Sf-9 cells tend to

utilize more Arg, Asn, Asp, Gln, Glu, Ser and Met than what is strictly required for incorporation into proteins.

In our present work, we have shown that from among the various amino acids, uninfected High-Five cultivated in YPR medium cells consume a lot of Asn, Gln (q_s ~ 30 µmol/10^9 cells.h) and little Arg, Asp, Gly, Glu, Lys, Thr (q_s ~ 2-10 µmol/10^9 cells.h) as energy source (AMFN, Figures 4-2). High-Five cells also consume little Arg and His as energy source (AMFN, Figure 4-2) while Sf-9 cells consume Ser (AMFN, Figure 4-5). On the other hand Gly, Ile, Leu, Met, Phe, Pro, Trp, Ser, Val are only required for the anabolism of High-Five cells (AMFN, Figure 4-2), and His, Gly, Ile, Leu, Met , Phe, Pro Trp, Tyr, Ser, Val for anabolism of Sf-9 cells (AMFN, Figure 4-4).

As a rule, both cell lines consume all of the essential amino acids for the production of biomass. Nevertheless, these cells consume also a few of them as energy source: Arg, His, Leu, Lys, Met and Thr for High-Five cells and Arg, Leu, Lys and Thr for Sf-9 cells. Among non-essential amino acids, all are highly consumed for both anabolism and catabolism except Ser that is not intensively used as energy source by High-Five and seems to be mainly used for the anabolism of High-Five cells.

4.4.1.2. Anabolism

A comparison of the consumption of nutrients by High-Five and Sf-9 cells and the production of ATP in exponential phase to those in stationary phase shows that the uninfected cells used nearly 3/4 of the ATP produced for their growth (biomass production) and a 1/4 for their maintenance (AMFN, Table 4-7). The demand of ATP per cell is higher in High-Five cells than in Sf-9 cells but the High-Five cells are larger than Sf-9 with a higher protein content. During growth phase, the demand of ATP (AMFN, Table 4-7) seems to be directly proportional to the cell protein biomass (Table 4-1) and seems well preserved in both Lepidopteran cell lines (High-Five and Sf-9). Based on the cell biomass composition, the growth rate, the specific production rate of ATP and by a simple calculation, we can establish that the ATP required by the cells for growth (cellular biomass synthesis) is respectively 197 mmol of ATP to produce 10^9 High-Five cells and 117 mmol/10^9 cells for Sf-9 cells (AMFN, calculated from Table 4-7). The maintenance rerquirements of High-Five and Sf-9 cells growing in YPR medium are, respectively, 1.11 and 0.70 mmol ATP/10^9cells.h.

4.4.2. METABOLISM OF INFECTED HIGH-FIVE AND SF-9 CELLS

During the infection phase, the metabolism of both High-Five and Sf-9 cells changes significantly. In this phase, the cells improve the use of available nutrients, and glucose is practically the only source of energy. Both High-Five and Sf-9 cells produce almost all of their ATP by glycolysis and the Krebs cycle (Table 4-6). On the other hand, by-products formation is similar as in the growth phase. During infection, the loss of energy due to the formation of by-products declines from 14 to 12 % for High-Five cells and from 24 to 15 % for Sf-9 cells (AMFN, calculated from Tables 4-6 and 4-7) compared to uninfected cells. During the infection phase, cells use most of the amino acids to synthesize proteins. The specific production rate of protein synthesis is similar to that during the growth phase. Nevertheless, the synthesis of cellular proteins for growth is negligible (AMFN, below 1 %) and the proteins produced are viral proteins, cellular proteins under the control of the virus replication and recombinant proteins. During infection, respectively 65 and 77 % of the proteins produced by High-Five and Sf-9 cells are represented by β-gal (AMFN, Table 4-7). The specific production rate of β-gal by High-Five cells is higher than that by Sf-9 cells but as stated above, the latter are smaller with a lower cell mass content (Ikonomou et al. 2003).

In a bioreactor context, the decrease of the specific rate of production of Sf-9 is partially compensated by the higher cell densities reached. Our data show that the specific rate of both biomass and recombinant protein synthesis is a function of the cellular biomass content (Tables 4-1 and 4-7). Infected Sf-9 cells use 81 % of the total amino acids consumed to synthesize proteins instead of 58 % for uninfected cells (AMFN, as can be evaluated from Table 4-7). The corresponding numbers for High-Five cells are 75 % for infected cells and 70 % for uninfected cells. In other words, Sf-9 cells are comparatively more efficient amino acid users than High-Five cells in their infection phase vis-à-vis their growth phase. The ATP level required to synthesize the recombinant protein is quite similar for High-Five and Sf-9 cells, i.e., respectively 169 and 136 μmol ATP/μg β-gal (AMFN, calculation based on the ATP production rate and on the specific production rate of β-gal form Table 4-7). During infection, the ATP used for maintenance is respectively 15 % for High-Five and 23 % for Sf-9 cells. During infection, Sf-9 cells consume lower levels of nutrients and produce less ATP than High-Five cells but Sf-9 cells produce less recombinant protein per cell than High-Five cells (AMFN, Table 4-7).

Several authors (as summarized by Palomares et al. 2004) have previously shown that the outcome of the infection is a function of the environmental culture conditions, the

cell lines and the baculovirus, among others. Moreover, the maximal productivity of recombinant protein is a function of the cell line, the cell density, the time of harvest and the MOI. We can now add to these observations that the maximal specific rate of production of recombinant protein and the nutrient consumption during infection is a function of the cell biomass content. Finally, the ATP required to synthesize the recombinant protein is directly proportional to the quantity of recombinant protein produced.

4.4.3. Metabolism of Asn in High-Five cells

Upon cultivation in YPR medium in exponential phase, High-Five cells consume more glucose, more Asn and less Gln than Sf-9 cells (Table 4-6) (Ikonomou et al. 2003). They also produce more lactate than Sf-9 cells (Table 4-5), in line with previous work (Rhiel et al. 1997). High-Five cells consume more Asn during lag phase and also when this amino acid is present at a higher concentration (Figures 4-3 and 4-4, Table 4-5). According to our work, and consistent with prior reports by Danner et al. (1995), Yang et al. (1996) and Rhiel et al. (1997), Asn is one of the most rapidly consumed amino acid in High-Five culture (data not shown). In 1997, Rhiel et al. proposed that the preferential use of Asn by High-Five could probably be due to higher activity of the enzymes involved in the metabolism of this amino acid. The consumption of this amino acid brings high energy to the cells (Table 4-6). Nevertheless, the consumption of amino acids by anaplerotic pathways of the Krebs cycle implicates an efflux of some organic acid(s) out the cycle. During the consumption of Asn and Gln the operation of the Krebs cycle is consistent with the efflux of malate and citrate out of the mitochondrion (AMFN, Figure 4-3). When High-Five cells are cultivated in high Asn medium compared to standard YPR medium, this externalisation doubles and produces 3.5 times more lactate, while the Ala production doubles (MSR, Table 4-5 and Figure 4-3).

The pathway of the use of Glu by Sf-9 was first studied by Öhman and coworkers in 1996 and was later clearly explained by Doverskog et al. (2000a). However, the case of Asn consumption by High-Five cells is not well studied until now. Based on our radiolabeled experiments (Table 4-8) and on the flows found in our network flux analysis (Figures 4-2 and 4-3), we can propose the following catabolic pathway of consumption of Asn, as shown in Figure 4-6 (this pathway represents a blow-up of a portion of the overall scheme in Figure 4-1: all enzymes and pathways present in Figure 4-6 are also present in Figure 4-1).

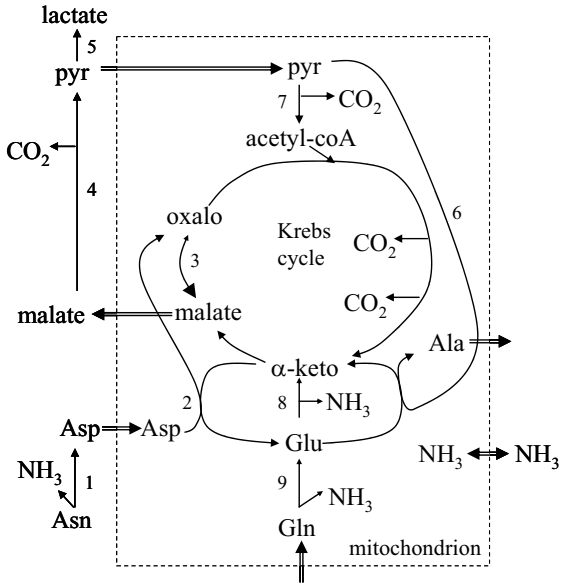


Figure 4-6 : Proposed metabolic network of asparagine in High-Five cells
Double lines represent the transfer of compounds from one cellular compartment to another. Full lines represent enzymatic pathways. Enzymes : 1, asparaginase; 2 aspartate aminotransferase; 3, malate dehydrogenase; 4, malic enzyme ; 5, lactate dehydrogenase; 6, alanine aminotransferase; 7, pyruvate dehydrogenase; 8, glutamate dehydrogenase; 9, glutaminase.

The Asn enters into the cell by a uniporter Asn/Asp (Murray et al. 1996; Wolfersberger 2000), where it is converted into Asp in the cytosol by an L-asparaginase (Figure 4-6: enzyme 1, Reaction 4-3). Asp goes across the mitochondrial membrane and is converted in the mitochondrion into oxaloacetate by an aspartate aminotransferase (Figure 4-6: enzyme 2, Reaction 4-4).

$$Asn \rightarrow Asp + NH_4^+ + H_2O \qquad \text{(Reaction 4-3)}$$

$$Asp + \alpha\text{-ketoglutarate} \rightarrow oxaloacetate + Glu \qquad \text{(Reaction 4-4)}$$

Oxaloacetate reacts with Acetyl-CoA (entering the Krebs cycle) (Figures 4-1 and 4-6) and thus leads to the production of CO_2 and ATP. Moreover, a part of oxaloacetate is converted to malate, either by malate dehydrogenase (AMFN, estimated between 2.5 to 25 % depending on whether High-Five cells are cultivated with 12 or 25 mM Asn, respectively), a reversible enzyme (Figure 4-6: enzyme 3, Reaction 4-6) or after a round of the Krebs cycle. Malate leaves the mitochondrion, and is converted to pyruvate by the cytosolic malic enzyme (Figure 4-6: enzyme 4, Reaction 4-6) (Gilmour 1961; Öhman et

al. 1996). Pyruvate could be used to produce lactate (Figure 4-6, Reaction 4-7), ethanol, alanine (Figure 4-6, Reaction 4-8) or Acetyl-CoA (Figure 4-6, Reaction 4-9), that may react with oxaloacetate in the Krebs cycle.

$$\text{malate} + NAD^+ \leftrightarrow \text{oxaloacetate} + NADH + H^+ \qquad \text{(Reaction 4-5)}$$

$$\text{malate} + NADP^+ \rightarrow \text{pyruvate} + CO_2 + NADPH \qquad \text{(Reaction 4-6)}$$

$$\text{pyruvate} + NADH + H^+ \leftrightarrow \text{lactate} + NAD^+ \qquad \text{(Reaction 4-7)}$$

$$\text{pyruvate} + \text{glutamate} \leftrightarrow \text{alanine} + \alpha\text{-ketoglutarate} \qquad \text{(Reaction 4-8)}$$

$$\text{pyruvate} + \text{CoA-SH} + NAD^+ \leftrightarrow \text{Acetyl-CoA} + CO_2 + NADH \qquad \text{(Reaction 4-9)}$$

During the operation of one turn of the Krebs cycle, decarboxylation removes one CO_2 from isocitrate (Figure 4-1: pathway 10) and one from α-ketoglutarate (Figure 4-1: pathway 11). The carbon atoms removed to produce CO_2 do not come from the acetyl-CoA in the first turn of the cycle (Lehninger et al.1993).

Subsequently, four CO_2 could be produce by oxaloacetate, either by four decarboxylations in the Krebs cycle (2 turnsof the cycle with 2 CO_2 produced per turn), or by the conversion of malate to pyruvate (one CO_2 produced, Reaction 4-5), by conversion of pyruvate to Acetyl-CoA (one more CO_2 produced, Reaction 4-9), by decarboxylation into the Krebs cycle (two additional CO_2 produced, Figure 4-1: pathways 10 and 11).
Based on these pathways and on a C atom mass balance (Equation 4-2), the total oxidation of one mole of Asn by the Krebs cycle produces 4 moles of CO_2, while the production from Asn of one lactate or one Ala (each one of them containing 3 carbon atoms), produces only one CO_2.

$$1\ \text{Asn}_{\text{consumed for catabolism}} \rightarrow a\ .\ \text{lactate} + b\ .\ \text{Ala} + \{\ 4\ .\ (1 - a - b) + (a + b)\ \}\ .\ CO_2 \qquad \text{(Equation 4-2)}$$

According to our data from the radiolabelled experiment (Table 4-8, Figure 4-3) and to this mass balance (Equation 4-2), we can calculated the expected production of $^{14}CO_2$ from the consumption of Asn^{14}C by High-Five cells. This calculated production of $^{14}CO_2$, from data of Table 4-8 and Equation 4-2, is equal to 3.04 moles of $^{14}CO_2$ per mole of Asn^{14}C consumed. Our experimental data of 2.74 moles of $^{14}CO_2$ trapped in hyamine per mole of Asn^{14}C consumed (Table 4-8) is close to this expected value and confirms the

above proposed pathway of consumption of Asn. Moreover, the lactate/alanine ratio expected in the analysis of the flux network (17.8 = 5.8/32.5, MSR, Figure 4-3) is close to the experimental value of lactate^{14}C/Ala^{14}C found (17.6= 0.3/1.7, Table 4-8) and confirms that lactate^{14}C and Ala^{14}C originate from the same malate^{14}C. Similar confirmations could be obtained with the other conditions (Table 4-8).

Concerning the regulation of this pathway, we can hypothesize that the high conversion of Asn into Asp in the cytosol increases the cytosolic concentration of Asp which decreases the gradient of concentration of Asp (between the cytosol and the medium) and inhibits the transport of Asp by uniporters into the cells (Murray et al. 1996; Wolfersberger 2000). In the mitochondrion, the high conversion of Asp into oxaloacetate increases the concentration of the latter. This favours the conversion of oxaloacetate into malate because the equilibrium of the malate-oxaloacetate reaction in the Krebs cycle (Reaction 4-5) is highly favourable to the production of malate (Lehninger et al. 2000). Furthermore, the high production of oxaloacetate by aspartate aminotransferase (Figure 4-6: enzyme 2, Reaction 4-4) accelerates the activity of the Krebs cycle by consuming α-ketoglutarate. The associated production of Glu, inhibits the consumption of Gln and explains why cells consume less Gln when fed with high Asn concentration (Figures 4-3 and 4-4). Moreover, Glu could be used to produce the deficient α-ketoglutarate by using alanine aminotransferase (that converts pyruvate into Ala) (Figure 4-6: enzyme 6, Reaction 4-8). Indeed, when High-Five cells consume high Asn, they produce more Ala (Figure 4-3, Tables 4-5 and 4-8). Besides, ammonia produced in the cytosol by the conversion of Asn to Asp, is in equilibrium with the mitochondrial ammonia which could inhibit the production of α-ketoglutarate by glutamate dehydrogenase (Figure 4-6: enzyme 8, Reaction 4-10) and the production of Glu by glutaminase (Figure 4-6:enzyme 9, Reaction 4-11) because both of them produce ammonia as by-product as pointed out previously for Sf-9 and EAA cells (Bédard et al. 1993).

$$Glu + NADP^{+} \rightarrow \alpha\text{-ketoglutarate} + NADPH + NH_3 \qquad \text{(Reaction 4-10)}$$

$$Gln + H_2O \rightarrow Glu + NH_3 \qquad \text{(Reaction 4-11)}$$

These latter observations support the various prior studies by us and others indicating that High-Five cells in lag phase consume more Asn, less glucose and Gln and produce more Ala. During the exponential phase, cells increase the consumption of glucose and Gln when the concentration of Asn in medium has decreased (Rhiel et al. 1997, Ikonomou et al. 2001, this work).

4.5. CONCLUSION

The use of a metabolic flux network is a good strategy to study in a holistic, interactive manner the complete metabolism of a cell line. The use of such a methodology requires the control of three key points: the construction of the network based on knowledge of existing studies on the metabolism of this cell lines, the accurate experimentation producing values of external flows (specific consumption/production rate of nutrients and by-products), and the good estimation of the flows of biomass production. The latter flows are based on the protein, DNA/RNA, lipid and carbohydrate content of the biomass and on the growth rate of cells.

We have shown that the use of a good estimation of these fluxes allows to study both the catabolism and the anabolism of a relatively less understood cell line, such as High-Five cells. The connection between the catabolism and anabolism can help to estimate the use of energy by the cells and the amount of nutrients required to synthesize their biomass.

Based on this flux network analysis, we found difference between the metabolism of the uninfected and baculovirus-infected High-Five and Sf-9 insect cells. Upon infection, cells used more glucose and less amino acids to produce ATP. During this phase, the majority of the ATP produced by the cells is used to synthesize the recombinant protein. This ATP used to synthesize the recombinant protein is constant and seems to be independent of the cell line. We also found that insect cells use glucose, Asn and Gln as energy source although High-Five cells prefer Asn, and Sf-9 Gln. We elucidated the metabolic pathway of the Asn in High-Five cells as follows: first Asn is converted in the cytosol to Asp; secondly Asp enters into the mitochondrion and feeds the Krebs cycle after being converted to oxaloacetate. We have confirmed quantitatively that insect cells waste energy by producing lactate and Ala. However, High-Five cells are able to consume the lactate and the Ala to provide energy when the other nutrients are deficient.

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III PART III: INSECT CELL PROCESSES

5

PART III: INSECT CELL PROCESSES

Chapter 5 : Enhancement of feeding strategies for protein production using the Insect Cell- Baculovirus System based on the analysis of cell metabolic requirements

5.1. INTRODUCTION

The development of the Insect Cell-Baculovirus Expression Vector System (IC-BEVS) two decades ago has enabled the widespread production of numerous heterologous proteins and viral pesticides and has brought industrial importance to insect cell culture (Agathos 1994). This transient expression system is essentially a co-culture of insect cells and baculovirus (Smith et al. 1983). In this system, insect cells are usually cultivated in batch culture and are infected with the baculovirus when they have reached a sufficient cell density before the stationary phase. Recombinant protein production by the IC-BEVS changes as a function of the cell line, the virus construction (Possee 1997) and the medium used (Schlaeger 1996a). Cell productivity is affected by various parameters. Cell density at infection and multiplicity of infection (MOI) (or number of virus per cell) are major factors (Chico and Jäger 2000). However the number of passages of the virus (Krell 1996), the "culture age" (Chico and Jäger 2000) and the state of medium depletion can also influence protein production (Ikonomou et al. 2003).

Today, the accruing knowledge in insect cell metabolism and vector construction together with the development of low-cost serum-free media and large-scale culture technology are creating a renewed industrial interest in insect cell systems. Nevertheless, as a rule, protein productivity decreases with increasing cell density (Chico and Jäger 2000). This is mainly due to nutrient depletion in the medium during infection. Thus, infection of batch culture is not convenient for high protein production levels. Usually a total change of medium is necessary before infection to ensure good productivity (Wu et al. 1998). Many systems exist to replace media. Unfortunately, many such methods may damage the cells, are costly and generally difficult to implement on a large scale (Ozturk 1996). Therefore, the recovery of high productivities on a large scale is a challenge. Different strategies have been developed to provide nutrients to cells without such a total changeover of medium. Perfusion cultivation is commonly used in industrial applications of animal cells and has already been applied to insect cells (Jäger 1996). The perfusion has the advantage of continuously removing metabolic by-products and bringing fresh nutrients to the cells. In the infection phase, this strategy allows the replenishment of nutrients to the cells and the continuous harvesting of the recombinant protein produced. The protein has a low residence time in the reactor with a low degradation and goes through a purification step (removal of cells and cell fragments)(Jäger 1996). Nevertheless, the use of high perfusion rates is costly in medium and dilutes the product titer (Ozturk 1996). Besides, the system of cell retention often causes high shear stresses and can damage the

cells whose membranes are already compromised by the virus infection (Palomares and Ramirez 1998). Moreover, in long-term perfusion, cell aggregation may appear (Ozturk 1996) and the extent of spontaneous mutation of the baculovirus increases with the number of replication cycles (Krell 1996). Thus, the perfusion culture of insect cells is better suited for the growth phase. Similar problems occur in the non perfused continuous culture of insect cells (i.e. without cell retention) with fewer advantages.

Fed-batch cultivation is an alternative technology that allows increasing the cell density without medium depletion. It is easier and cheaper to implement than perfusion, avoids exposure of the cells to high shear stress in a cell retention system but requires knowledge of cell physiological requirements. Fed-batch cultivation of insect cells has been studied intensively in recent years, especially in an effort to increase the cell density of *Spodoptera frugiperda* cell lines. Reuveny et al. (1994) obtained 15.8×10^6 Sf-9 cells/ml in IPL-41 serum-free medium by a combination of medium replacement and fed-batch. Chan et al. (1998) searched to optimize the production of recombinant β-gal with Sf-9 cells infected in fed-batch between densities of 4 and 12×10^6 cells/ml. Chiou et al. (2000) have cultivated the Sf-21 cell line and reached 19×10^6 cells/ml in TNM-FH medium with 10 % foetal bovine serum (FBS). Kim et al. (2000) studied the infection of Sf-9 cells in Grace's serum-free medium supplemented with amino acids and yeast extract. Cultivated in serum-free SF900-II medium in a fed-batch configuration, Sf-9 cells reached 30×10^6 cells/ml and were infected successfully at 11.5×10^6 cells/ml (production of 895 U β-gal / ml) (Bédard et al. 1994; Bédard et al. 1997). Elias et al. (2000) improved the previous work and reached 52×10^6 cells/ml which is the highest cell density reported, in fed-batch mode, so far with lepidopteran cells. Nevertheless, the decrease of specific productivity with increasing cell density did not allow infection at such a cell density and Elias et al. (2000) used a maximal cell density of 17×10^6 cells/ml for subsequent infection. Other efforts in this area include a structured model of batch or fed-batch culture of Sf-9 to study cell metabolism (Jang et al. 2000) and a fed-batch two-stage reactor system for Sf-21 cells cultivated in a poor medium with 10 % FBS reaching $1\text{-}2 \times 10^6$ cells/ml (van Lier et al. 1996). Concerning the *Trichoplusia ni* High-Five cell line, Wu et al. (1998) attained 4×10^6 cells/ml and infected the cells in IPL-41 medium with 10 % FBS. Generally, fed-batch cultivation reports have studied the increase of production and/or cell density but some articles have addressed the use of fed-batch to study insect cell metabolism (Nguyen et al. 1993; Öhman et al. 1995). Recently, Martejin et al. (2003) proposed a genetic algorithm to optimise the culture media in a fed-batch of a *Helicoverpa zea* cell line.

As can be seen from the reports cited above, until now fed-batch culture of insect cells has been studied mainly for *Spodoptera frugiperda* lines and has aimed to increase the cell density and not to improve the infection in serum-free media. The aim of this work was to design a fed-batch supplementation strategy for optimal exploitation of the production potential of the High-Five cell line. To date, these cells have not been adequately studied in fed-batch for production, except for the work by Wu et al. (1998). In batch culture, these cells are able to grow in serum-free media and in general have a higher productivity than Sf-9, hence High-Five cells are considered promising for industrial application.

We report here the development of a fed-batch culture strategy aimed at protein production by High-Five cells, in comparison with Sf-9 cells. First, we sought to evaluate the cell requirements, during the growth phase, by factorial experiments, using several mass balances and supplementation combinations. Secondly, we tried to extrapolate this knowledge to the infection phase by testing different supplement combinations, whose effects lead finally to enhanced protein production. In contrast to other works, we did not search to obtain high cell densities but high cell productivities with the simplest feeding strategies.

5.2. MATERIALS AND METHODS

5.2.1. Cell lines, Viruses

The insect cell lines used in this research were *Trichoplusia ni* High-Five and *Spodoptera frugiperda* Sf-9. The High-Five cells were a gift of the Laboratory of Virology (University of Wageningen, The Netherlands), whereas Sf-9 were bought from Invitrogen (Carlsbad, CA). Two recombinant constructs of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) were used, one encoding β-galactosidase (β-gal) (Invitrogen) and the other expressing human secreted placental alkaline phosphatase (seAP) which was donated by H.A. Wood (Boyce Thompson Institute of Plant Research, Ithaca, NY).

5.2.2. Medium, additives

Cell growth was examined in YPR, a serum-free medium developed in our laboratory (Ikonomou et al. 1999) to which dextran was added. This medium is composed of IPL-41 medium (Hyclone, Logan, UT) supplemented with 41 mM glucose, 3.5 mM glutamine (to obtain, respectively, final concentrations of 55 and 10 mM), 4 mM $NaHCO_3$, 0.1 % (v/v) of Pluronic F-68, 0.25 % (v/v) of lipid mixture for insect cell culture (Sigma,

St. Louis, MO), 1 g/l of dextran 67 kDa (Sigma) and the ultrafiltered hydrolysates Yeastolate (a yeast extract) at 6 g/l (Sigma) and Primatone at 5 g/l (Kerry Bio-Science, Almere, The Netherlands), prepared with milli-Q water (Millipore, Molsheim, France), pH 6.2–6.3. Cocktails tested (used as 5X concentrated) in our experiments were BME (Basal Medium Eagle's) vitamins solution from Sigma, MEM (Miminum Essential Medium) essential amino acids mixture solution without L-glutamine and MEM non-essential amino acids solution from Gibco (Invitrogen). Lactalbumin and Tryptose Broth Phosphate hydrolysates tested were from Sigma.

5.2.3. Culture conditions

Experiments were performed in duplicate in shake flasks of 125 and 250 ml (Corning, Acton, MA) at 28°C and 130 rpm with respectively 25 and 50 ml of medium. Suspension-adapted High-Five™ cells were inoculated at $0.2x10^6$ cells/ml and Sf-9 cells at $0.4x10^6$ cells/ml.

Bioreactor studies were carried out in a Celligen-Plus™ bioreactor (New Brunswick Scientific, Edison, NJ). The glass bioreactor was siliconized with Sigmacote (Sigma) to prevent cell adhesion. The dissolved oxygen level was maintained at 50 % of air saturation by headspace aeration whereas for agitation a pitched blade impeller was used between 70 and 100 rpm. A dynamic measure of oxygen consumption rate by the cells was performed daily in the course of the cultivation.

Cell density and viability were estimated by Trypan Blue dye exclusion (0.2 % w/v final concentration). Analyses of glucose, lactic acid, ammonia and pH were done using a Bioprofile 100 Analyser (Nova Biomedical, Waltham, MA). Glutamine (Gln) and glutamate (Glu) were determined by a glutamate enzymatic kit (Boehringer, Mannheim, Germany). Glu and Gln samples were analysed separately. Before analysis by the kit, Gln samples (50 µl) were treated with 10 µl asparaginase (400 units/ml, Sigma) in order to transform completely Gln to Glu (37°C, 30 min in PBS buffer). Then the sum of Glu plus Gln was determined. Gln was determined by subtraction of the previously determined Glu.

The other amino acid samples were deproteinized with sulfosalicylic acid and analysed by high-pressure liquid chromatography (HPLC) after *o*-phthaldialdehyde (OPA) derivatization using a Spherisorb ODS column (Waters, Brussels, Belgium) as described by Fekkes et al. (1995).

5.2.4. STUDIES OF FEEDING STRATEGIES

For fed-batch studies, cells were inoculated in YPR medium and were grown in batch before adding different fed-batch supplements to increase cell growth and to extend the stationary phase. Batch cultivations were used as controls in fed-batch studies.

For infection experiments, we inoculated cells in YPR medium and added supplements at different times before and/or during infection. Experiments were performed with either a β-gal or a seAP baculovirus and a MOI of 2. Infections of batch cultures after total changeover of medium served as positive controls. Infections of batches without any change of medium or fed-batch addition served as negative controls. The samples of both the intra- and the extracellular recombinant protein produced were assayed according to Miller (1972) and Dee et al. (1997) respectively for β-gal and seAP enzymatic activities.

In order to keep similar shear stress and oxygen transfer to cells, all supplementation experiments were done at constant volume. Before the addition of supplements for fed-batch experiments to flasks, the same volume of liquid was removed for sampling and to keep the culture volume constant. We normalized production to take into account the sampling volume. In order to compare production between conditions independently of the supplementation, we used total production of cells (U/flask) at the end of the cultivation (144 h.p.i.): volume of culture (ml/flask) multiplied by the total volumetric production level (intra- and extracellular) (U/ml). The yield of production is defined as the total production (U/flask) divided by the total number of viable cells (10^6 cells/flask) in the same flask at the point of infection (0 h.p.i.).

To facilitate the readability of the data, the following notation of the different supplement formulations is used: Ingredients are represented by letters: M for medium, G for glucose, Gln for glutamine, L, P, T and Y respectively for Lactalbumin, Primatone, Tryptose broth phosphate and Yeastolate hydrolysates. Numbers in subscripts indicate concentration in mM for glucose and Gln and in g/l for Yeastolate and Primatone. The subscript on M indicates the concentration factor (strength) of all medium components except G, Gln, P and Y. Whenever nutrients are dissolved in water, M is omitted. In these notations, a supplement equivalent to YPR medium is denoted as $M_1G_{55}Gln_{10}P_5Y_6$.

5.3. RESULTS

5.3.1. FED-BATCH DURING CELL GROWTH PHASE

5.3.1.1. Factorial experiment

The first step in the formulation of our fed-batch supplement was to determine which compounds could increase cell growth and extend the stationary phase of insect cells. Glucose addition was justified by its high consumption rate by insect cells and its importance during infection. Gln was suggested for its importance in sustaining high growth rate (Ikonomou et al. 2001). Different hydrolysates were tested for their capacities to increase cell growth (Nguyen et al. 1993; Schlaeger 1996b).

We chose to test a feed equal to 20 % of the culture volume. The use of one fifth of the culture volume prevents excessive dilution of cell density and allows convenient preparation of the supplement. Moreover, this addition should provide enough nutrients to the cells for 2 days after addition (Ikonomou et al. 1999).

For this first screening test, two factorial experiments (FE) were done with High-Five cells in growth phase, a complete FE 2^{6-2} (for G, Gln, P, Y, L, T) (data not shown) and a reduced FE 2^{4-1} (for G, Gln, P, Y) (Tables 5-1 and 5-2).

Table 5-1: Factorial experiment for the screening of fed-batch supplement formulations.

Supplement formulation				High-Five cell density (10^6 cells/ml) and viability (%)		
Glucose 140 mM	Glutamine 25 mM	Primatone 12.5 g/l	Yeastolate 12.5 g/l	0 h	17 h	48 h
+	-	-	+	4.2 (98 %)	4.7 (89 %)	3.8 (92 %)
+	-	+	-		3.5 (85%)	5.2 (93 %)
-	+	+	-		4.0 (96 %)	2.0 (95 %)
+	+	-	-		4.0 (92 %)	2.8 (84 %)
-	+	-	+		4.9 (92%)	3.1 (87 %)
-	-	+	+		4.4 (92 %)	4.5 (95 %)
+	+	+	+		5.1 (88 %)	5.2 (91 %)
-	-	-	-		3.6 (91 %)	3.0 (88 %)

After cultivation in batch, the cells received a single fed-batch addition (20 % of culture volume). The supplements were diluted in water and their final concentrations are indicated. Each + and - indicates the presenc e or absence of a compound in the supplement. The control (last line) was a 20% of culture volume addition, consisting of water.

Table 5-2: Effects of each supplemented compound on the acceleration of the growth phase and the extension of the stationary phase of batch-grown High-Five cells in the FE 2^{4-1} experiment (Table 5-1). The separate effects of each compound have been estimated according to Montgomery (1997).

Ingredient	Acceleration of the growth phase	Extension of stationary phase
Glucose	+	+++
Glutamine	+++	--
Primatone	0	+++
Yeast extract	++	--

Lactalbumin and Tryptose Broth Phosphate gave poor results and were abandoned (data not shown). We observed a contrast between compounds that tended to increase the growth phase and those that extended the stationary phase (Tables 5-1 and 5-2). Gln increased most significantly the growth on the first day after addition but had a negative effect over the next days. The most pronounced effect of glucose was to extend the stationary phase. The two hydrolysates had distinct effects: Yeastolate sustained the growth whereas Primatone extended the stationary phase.

5.3.1.2. Glucose requirement

We decided at first to examine the cells' glucose consumption. After batch cultivation of High-Five cells, when 4.0 x 10^6 cells/ml were reached, we added different nutrient combinations: one single addition (20 % of initial volume) of $G_{140}Gln_{10}P_{10}Y_{10}$, $M_1G_{70}Gln_{10}P_5Y_6$ and $M_1G_{55}Gln_{10}P_5Y_6$. The residual concentrations of glucose at the end of the cultivations were, respectively, 17.8 mM, 2 mM and 0 mM (data not shown). A mass balance indicated that the consumption of glucose by the High-Five cells in the 48 hours after addition was around 1.25-1.5 mmol/10^9 cells.day (data not shown). In order to be sure that the cells would have enough glucose throughout the duration of the fed-batch cultivation, we adjusted the glucose concentration in the supplement at 70 mM.

5.3.1.3. Gln and Asn requirement

Next, we examined Gln consumption. Three different concentrations of Gln (10 mM, 20 mM and 40 mM) were tested in a fed-batch supplement (20 % of culture volume consisting of $M_1G_{70}P_{10}Y_{10}$) when High-Five cells had reached a density of 4.0x10^6 cells/ml (data not shown). The residual concentration of Gln two days after these additions was below 0.5 mM for Gln_{10}, 2.5 mM for Gln_{20} and 5.5 mM for Gln_{40}. We chose to retain the concentration of 20 mM because this level assured better growth than 10 mM (level at

which the cells were lacking Gln) whereas 40 mM brought no improvement on growth and increased unnecessarily the residual Gln concentration.

Although asparagine is the most rapidly consumed amino acid by High-Five cells (Rhiel et al. 1997), we found that its addition was not required to increase cell density (data not shown).

5.3.1.4. Lipid and vitamin requirements

After these determinations, we tested further potential requirements of minor compounds of the medium, including amino acids, lipids and vitamins (Table 5-3). In the presence of $G_{70}Gln_{20}P_{10}Y_{10}$, the addition of the cocktail of essential amino acids was beneficial for growth. Lipids had a slight negative effect on growth. Effects of vitamins were positive but less consequential towards increasing the cell density compared to amino acids. However, any one of these three solutions in water allowed to reach a cell density comparable to that obtained with the addition of this new supplement diluted in medium solution with a strength factor of 1 ($M_1G_{70}Gln_{20}P_{10}Y_{10}$).

Table 5-3: Analysis of the effects of amino acids, lipids and vitamins on High-Five cell growth.

Supplement formulation	Cell density (10^6 cells / ml) and viability (%)	
	0 h	26 h
Basal supplement + amino acids + lipids	3.4 (98 %)	5.1 (94 %)
Basal supplement + amino acids + vitamins		5.6 (96 %)
Basal supplement + lipids + vitamins		4.9 (95 %)
Basal supplement + amino acids + lipids + vitamins		5.3 (95 %)
Basal supplement diluted in medium instead of water		5.8 (96 %)
Medium (YPR : $M_1G_{55}Gln_{20}P_5Y_6$)		4.9 (95 %)
Water (control)		4.0 (96 %)

Identical batches of High-Five cell culture received the different supplements in a single addition equivalent to 20 % of culture volume. The supplements were diluted in water and the basal supplement composition was G 70 mM, Gln : 20 mM, P and Y : 10 g/l each , plus, for the different conditions : the MEM essential amino acid solution ("amino acids"), BME vitamins solution ("vitamins") and the lipid mixture ("lipids").

The addition of a cocktail of non-essential amino acids (data not shown) brought no improvement compared to $M_1G_{70}Gln_{20}P_{10}Y_{10}$, i.e., the dissolution of the supplement in medium was adequate. Therefore, we tried to explore different medium strengths (0.5 and 1) for the supplement formulation $G_{70}Gln_{20}P_{10}Y_{10}$ and a control in water. The strength of 1 had a better effect than that of 0.5 which was still more concentrated than the control

(data not shown). These separate additions gave poor results because cells needed all of these nutrients together (Tables 5-1 and 5-3). Further, we hypothesized that enriched medium could give better results, since the major improvements were already reflected in the effects of the hydrolysates.

5.3.1.5. Fed-batch cultivation of High-Five and Sf-9 cells

We tried to concentrate the supplements for fed-batch cultivation by dissolving all compounds plus the medium powder in one-half the amount of water usually employed ($M_2G_{140}Gln_{40}P_{20}Y_{20}$), or by doubling the concentration of glucose, Gln and hydrolysates ($M_1G_{140}Gln_{40}P_{20}Y_{20}$).

We added successive pulses of supplements (each pulsed addition representing 20% of the initial culture volume) every day of cultivation to High-Five cells in mid exponential phase (Figure 5-1). The two first additions of these formulations ($M_2G_{140}Gln_{40}P_{20}Y_{20}$, $M_1G_{70}Gln_{20}P_{10}Y_{10}$ and $M_1G_{140}Gln_{40}P_{20}Y_{20}$) increased the High-Five cell density. Unfortunately, the formulation $M_2G_{140}Gln_{40}P_{20}Y_{20}$ (700 mOsm/kg) caused an increase of medium osmolality from 350 to 420 mOsm/kg which is deleterious for the cells (Schlaeger 1996a) and the formulation $M_1G_{140}Gln_{40}P_{20}Y_{20}$ caused a high production of lactate. For all conditions, the final (fourth) pulses caused dilution of cells and were deemed unnecessary. Hence, based on these previous observations (subsections 5.3.1.1 to 5.3.1.5), the best supplement sustaining the growth of High-Five cells is a single addition of 20 % of $M_1G_{70}Gln_{20}P_{10}Y_{10}$, the less concentrated supplement.

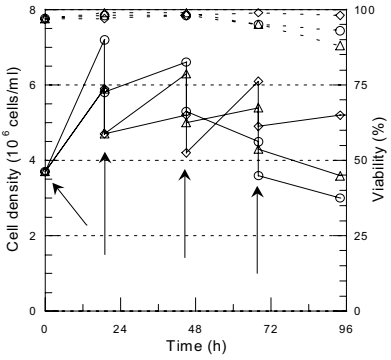

Figure 5-1: Time course of cell density (full lines) and viability (dashed lines) when High-Five cells were fed with $M_1G_{70}Gln_{20}P_{10}Y_{10}$ (◊), $M_1G_{140}Gln_{40}P_{20}Y_{20}$ (Δ) or $M_2G_{140}Gln_{40}P_{20}Y_{20}$ (○). Arrows indicate the timing of supplement addition, each time at 20 % of the initial culture volume.

Concerning Sf-9, similar experiments were done. We added successive pulses of supplements every 24 hours to Sf-9 cells in batch culture (Figure 5-2), each pulse representing 25 % of the initial culture volume, in order to sustain a high Sf-9 cell density. We observed that growth continued after the second addition of supplements. Little lactate accumulation occurred during fed-batch (< 5 mM) and the specific glucose consumption rate remained stable. In contrast to High-Five cells, glucose consumption decreased with the number of pulsed additions (data not shown). The highest cell density (25.10^6 cells/ml) was obtained with three pulses of $M_1G_{140}Gln_{40}P_{20}Y_{20}$. However, the best growth rate was observed with two pulses of 25 % of culture volume in the form of $M_1G_{70}Gln_{20}P_{10}Y_{10}$.

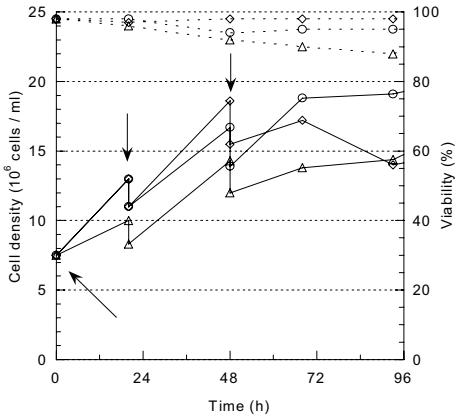

Figure 5-2: Time course of cell density (full lines) and viability (dashed lines) when Sf-9 cells were fed with $M_1G_{70}Gln_{20}P_{10}Y_{10}$ (◊), $M_1G_{140}Gln_{40}P_{20}Y_{20}$ (Δ), $M_2G_{140}Gln_{40}P_{20}Y_{20}$ (○). Arrows indicate the timing of supplement addition, each time at 25 % of the initial culture volume.

5.3.2. FED-BATCH DURING INFECTION PHASE

5.3.2.1. Feeding strategies for infection of High-Five cells

At first, we researched the quantity of supplement suitable for the cells and the frequency of addition. In a preliminary experiment, we infected High-Five cells in mid growth phase ($3.3x10^6$ cells/ml) with different pulses of $M_1G_{70}Gln_{20}P_{10}Y_{10}$. An addition of 40 % of the culture volume in one or two pulses produced respectively 410 and 680 U β-gal /ml, as opposed to the positive control (total medium replacement with fresh YPR medium) which produced 1400 U β-gal / ml (data not shown). Hence, for the same amount of nutrient supply, cells fed with several additions produce more than cells receiving a single addition.

On the basis of these indications, we decided to improve these additions. For infection of High-Five batches (Table 5-4 part A and Figure 5-3), the additions of 3 x 20 % (i.e., three pulses of 20% culture volume equivalents) or 6 x 10 % of $M_1G_{70}Gln_{20}P_{10}Y_{10}$ sustained similar productions (592 and 619 U β-gal / 10^6 cells). This indicates that, at equal quantity of nutrients, it was sufficient to dispense supplement every two days.

Table 5-4: Production of recombinant protein using different feeding strategies for infection of insect cells previously grown in batch cultures. The cells were cultivated in batch and received the supplements solely during the infection as shown.

A. Infection of High-Five batches

Cell density at infection (10^6 cells / ml)	Supplement formulation	Feeding strategy used (quantity and frequency of addition) after infection	Total production (U β-gal / flask)	Yield (U β-gal / 10^6 cells)
4.6	Positive control (total medium change-over at infection)		66090	574
4.6	Negative control (without any medium change-over)		15770	137
4.6	I	3 x 20 % vol (0, 48 and 96 hpi)	68140	592
4.6	I	6 x 10 % vol (0, 24, 48, 96 and 120 hpi)	71200	619
4.6	II	3 x 10 % vol (0, 48 and 96 hpi)	42640	370
4.6	II	3 x 20 % vol (0, 48 and 96 hpi)	63500	552
4.6	III	3 x 10 % vol (0, 48 and 96 hpi)	47430	412
4.6	III	3 x 20 % vol (0, 48 and 96 hpi)	75600	657

Supplement I: $M_1G_{70}Gln_{20}P_{10}Y_{10}$, Supplement II: $M_1G_{140}Gln_{40}P_{20}Y_{20}$,
Supplement III: $M_2G_{140}Gln_{40}P_{20}Y_{20}$

B. Infection of Sf-9 batches

Cell density at infection (10^6 cells / ml)	Supplement formulation	Feeding strategy used (quantity and frequency of addition) after infection	Total production (U β-gal / flask)	Yield (U β-gal / 10^6 cells)
8.6	Positive control (total medium change-over at infection)		18130	84
8.6	Negative control (without any medium change-over)		2240	10
8.9	I	3 x 25 % vol (0 ,48 and 96 hpi)	32940	148
8.9	I	6 x 25 % vol (0, 24, 48, 96 and 120 hpi)	29450	132
8.9	II	3 x 25 % vol (0 ,48 and 96 hpi)	27680	124
8.9	II	6 x 25 % vol (0, 24, 48, 96 and 120 hpi)	20750	93
8.9	III	3 x 25 % vol (0 ,48 and 96 hpi)	5490	25
8.9	III	6 x 25 % vol (0, 24, 48, 96 and 120 hpi)	5350	24

Supplement I: $M_1G_{70}Gln_{20}P_{10}Y_{10}$, Supplement II: $M_1G_{140}Gln_{40}P_{20}Y_{20}$,
Supplement III: $M_2G_{140}Gln_{40}P_{20}Y_{20}$

At equal quantity of nutrients (Table 5-4 part A), the β-gal production using 30 % (3 x 10 %) of $M_2G_{140}Gln_{40}P_{20}Y_{20}$ was less than that obtained with the same feeding schedule but less concentrated supplement: 60 % (3 x 20 % or 6 x 10 %) of $M_1G_{70}Gln_{20}P_{10}Y_{10}$ (a less concentrated supplemented). This appears to have been caused by the increase of osmolality. Moreover, the feeding strategy involving $M_2G_{140}Gln_{40}P_{20}Y_{20}$ led to nutrient accumulation in the culture, whereas with the supplement $M_1G_{70}Gln_{20}P_{10}Y_{10}$ neither excessive glucose and amino acids additions nor excessive accumulation of lactate and ammonia were observed (Figure 5-3). Hence, we retained this supplement ($M_1G_{70}Gln_{20}P_{10}Y_{10}$) for the subsequent improvement steps.

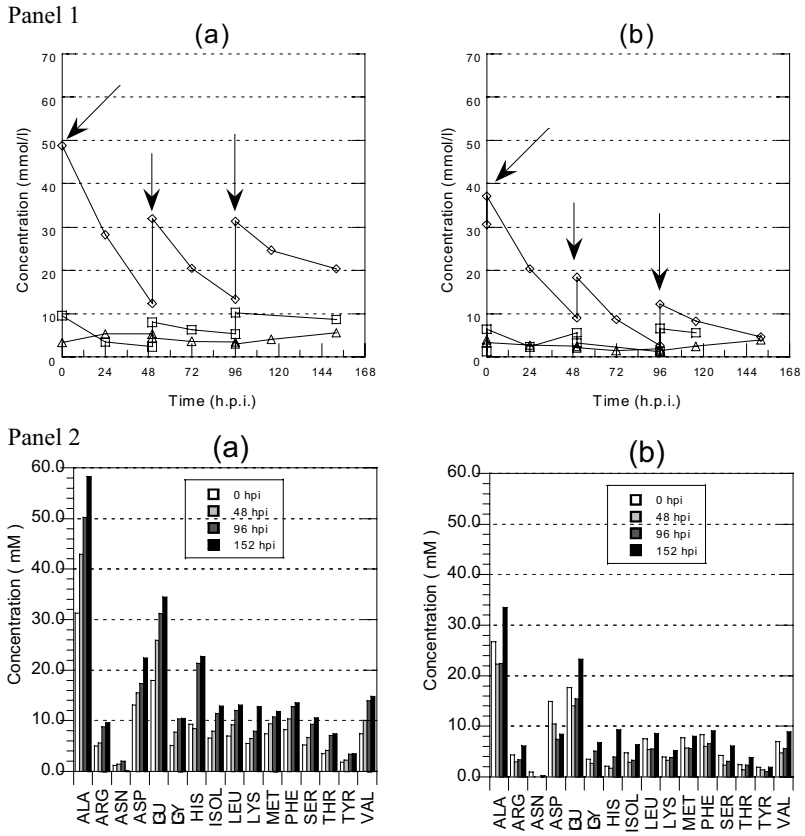

Figure 5-3: Infection of High-Five cell batches at a density of $4.6x10^6$ cells/ml using different supplementation strategies with three additions of 20 % culture volume equivalents of $M_2G_{140}Gln_{40}P_{20}Y_{20}$ (supplement III) (panel a) or $M_1G_{70}Gln_{20}P_{10}Y_{10}$ (supplement I) (panel b). Time course of metabolites (panel 1), glucose (◊), glutamine (□), lactate (Δ) and amino acids (panel 2). Arrows indicate additions.

This feeding strategy was tested in a Celligen-Plus bioreactor cultivation: after growth, High-Five cells were infected in the reactor at $4.5x10^6$ cells/ml. They received three pulses of 150 ml of $M_1G_{70}Gln_{20}P_{10}Y_{10}$ during the course of the infection phase (data not shown). Protein titre in the bioreactor was similar (1650 U β-gal / ml at 168 h.p.i.) as in the shake-flask control under the same conditions (1570 U β-gal / ml at 144 h.p.i.), with respective yields of 650 and 625 U β-gal / 10^6 cells.

Encouraged by these data, we next attempted to infect fed-batch cultivated High-Five cells at higher density. Fed-batch cultures received $M_1G_{70}Gln_{20}P_{10}Y_{10}$ (20 % of culture volume) during the growth phase to obtain ca. 6 x 10^6 cells/ml before infection and were subjected to various combinations of supplements during infection: 3 and 6 x 20 % of $M_1G_{70}Gln_{20}P_{10}Y_{10}$, $M_1G_{140}Gln_{40}P_{20}Y_{20}$ and $M_2G_{140}Gln_{40}P_{20}Y_{20}$ (data not shown). Unfortunately, these infections of fed-batch cultures, produced less β-gal and accumulated more ammonia and lactate by-products than the previous infections of batch cultures (Table 5-4, part A).

5.3.2.2. Feeding strategies for infection of Sf-9 cells

Batches of Sf-9 cells were infected at ca. $9x10^6$ cells/ml at the end of the mid-growth phase (Table 5-4 part B). Based on these cells' tendency for high nutrient consumption, we chose to add several pulses of 25 % culture volume equivalents. The production sustained by the less concentrated supplement ($M_1G_{70}Gln_{20}P_{10}Y_{10}$) was better than with the more concentrated supplements.

For batch infection, at equal quantity of nutrients added, the β-gal production using 3 x 25 % of supplement $M_2G_{140}Gln_{40}P_{20}Y_{20}$ in the course of infection is not as effective as 6 x 25 % of $M_1G_{70}Gln_{20}P_{10}Y_{10}$ due to the high increase of osmolality (Table 5-4 part B). The addition of 3 x 25 % of supplement $M_1G_{70}Gln_{20}P_{10}Y_{10}$ was sufficient to obtain a good production.

In contrast to High-Five cells, the lactate production of Sf-9 cells was below the detection limit (< 1 mM) (data not shown). However, we observed a high residual concentration of glucose and Gln under all conditions tested (i.e., between 34 and 89 mM for glucose and between 10 and 35 mM for Gln, for the conditions given in Table 5-4, part A) (data not shown).

Next, we attempted to infect Sf-9 fed-batch cultures at higher cell densities (Table 5-5). We cultivated Sf-9 cells adding the best growth sustaining supplement ($M_1G_{70}Gln_{20}P_{10}Y_{10}$ at 1 or 2 x 20 %) during the growth phase (Table 5-5), while during infections different patterns of supplementation were tested. At equal quantity of

supplements, infections done at $12.8x10^6$ cells/ml gave higher production titer than at $15.6x10^6$ cells/ml. Addition of 150 % (6 x 25 %) of supplement $M_1G_{70}Gln_{20}P_{10}Y_{10}$ during the infection sustained the higher productivity (Table 5-5), but with an excessive addition of nutrients (data not shown). A mass balance indicated that use of 100 % should prevent this accumulation (either by addition of 4 x 25 % or 5 x 20 %).

Table 5-5: Production of recombinant protein using different feeding strategies for infection of Sf-9 insect cells previously grown in fed-batch cultures

Cell density at infection (10^6 cells / ml)	Supplement formulation	Feeding strategy used after infection (quantity and frequency of addition)	Total production (U β-gal / flask)	Yield (U β-gal / 10^o cells)
12.8	Positive control (total medium change-over at infection)		15680	48
12.8	Negative control (without medium change-over at infection)		850	3
12.8	I	3 x 25 % vol (0 ,48 and 96 hpi)	16220	50
12.8	I	6 x 25 % vol (0, 24, 48, 96 and 120 hpi)	31590	98
12.8	II	3 x 25 % vol (0 ,48 and 96 hpi)	21220	66
15.6	Positive control (total medium change-over at infection)		5740	15
15.6	Negative control (without any medium change-over at infection)		620	2
15.6	I	3 x 25 % vol (0 ,48 and 96 hpi)	8760	22
15.6	I	6 x 25 % vol (0, 24, 48, 96 and 120 hpi)	32110	82
15.6	II	3 x 25 % vol (0 ,48 and 96 hpi)	25010	64

Supplement I : M_1G_{70} [illegible] $_{20}P_{10}Y_{10}$; Supplement II: M_1G_{140} [illegible] $_{40}P_{20}Y_{20}$

Sf-9 cells were cultivated in batch culture in YPR medium and received either one (24 h before infection) or two additions (24 and 48 h before infection) of supplement M_1G_{70} [illegible] $_{20}P_{10}Y_{10}$ at 20 % of culture volume, before infection took place, respectively, at a cell density of 12.8 or 15.6 x 10^6 cells / ml. Cells were supplemented during the infection as shown in the Table.

5.3.2.3. Enhancement of feeding strategies for High-Five and Sf-9 cells

Infections of High-Five cultures at a density around 4-5x10^6 cells/ml was the better choice (Table 5-4 part A, Figure 5-3). The previous results indicate that three additions of 20 % of culture volume equivalent in the form of supplement $M_1G_{70}Gln_{20}P_{10}Y_{10}$ was the better condition for high protein production, but not yet optimized. In such conditions, we added 3 x 20 % of $M_1G_{70}Gln_{20}P_{10}Y_{10}$, that is 42 mmoles/l of glucose and 6 mmoles/l of Gln added during the infection. To improve the addition as a function of cellular requirements, we decided to keep the same overall quantity of glucose but with a gradual reduction of concentration adapted to the cells' needs (Figure 5-3) from 100 mM (at the time of infection) to 40 mM (for the last addition). Concerning glutamine, we found that High-Five

cells needed Gln only in the two first additions (Figure 5-3). We chose to keep the same overall quantity of Gln by the addition of 4 mmoles/l at the time of infection, 2 mmoles/l at the second addition and no Gln at all in the last addition (Table 5-6).

Table 5-6: Improved feeding strategies for infection of High-Five batches

Feeding strategy used (quantity, supplement formulation and frequency of addition)			Total production (U β-gal / flask)	Yield (U β-gal / 10^6 cells)	Total production (U seAP / flask)	Yield (U seAP / 10^6 cells)
0 hpi	48 hpi	96 hpi				
Positive control (total medium change-over at infection)			59320	527	1980	17.6
Negative control (without any medium change-over)			13780	122	525	4.6
20 % vol of supplement IV	20 % vol of $G_{70}Gln_{10}$ + $M_1P_{10}Y_{10}$	20 % vol of G_{40} + $M_1P_{10}Y_{10}$	56080	498	1840	16.4
20 % vol of supplement IV	20 % vol of $G_{70}Gln_{10}$ + M_1	20 % vol of G_{40} + M_1	77200	686	2880	25.6
20 % vol of supplement IV	20 % vol of $G_{70}Gln_{10}$ + $P_{10}Y_{10}$	20 % vol of G_{40} + $P_{10}Y_{10}$	55320	492	1920	17.0
20 % vol of supplement IV	20 % vol of $G_{70}Gln_{10}$	20 % vol of G_{40}	45360	403	1520	13.5

Supplement IV : M_1G_{100} $_{20}P_{10}Y_{10}$

Batches of High-Five cells were infected at 4.5 x 10^6 cells / ml. After a uniform addition of 20 % vol at 0 hpi of supplement IV (M_1G_{100} $_{20}P_{10}Y_{10}$), different supplement formulations were tested for the two last additions (48 and 96 hpi). Experiments were performed with a β-gal and a seAP baculovirus.

Infections of Sf-9 in fed-batch cultures at a density around 12-13x10^6 cells/ml was the better choice (Table 5-4 part B, Table 5-5). The previous results indicate that additions of 100 % of culture volume of supplement $M_1G_{70}Gln_{20}P_{10}Y_{10}$ was the better condition for high protein titer, but not yet optimized. For further enhancement, we adopted an addition of a volume of 5 x 20 % (Table 5-7). The overall quantity of glucose needed by the cells during infection was 45 mmoles/l. Based on this glucose mass balance, we retained the addition of this quantity in five pulses ranging in glucose concentration from 70 down to 20 mM for the enhancement of feeding. With regard to Gln, we decided to add the five pulses in a range from 20 down to 5 mM (Table 5-7).

The hydrolysates were useful for infection of the two cell lines but their concentration had to be improved. We tested various combinations of supplements for the infection of High-Five (Table 5-6) and Sf-9 cells (Table 5-7) by varying concentration of hydrolysates and strength of medium. For the infection of High-Five batches (Table 5-6),

we observed a better production with a medium strength of one both for seAP and β-gal. The presence of Primatone and Yeastolate plus medium at the end of the infection seems to be unfavorable (Table 5-6). Their presence appears positive compared to the addition of only glucose and Gln. For the best condition obtained (492 U β-gal and 17 U seAP /10^6 High-Five cells), the residual concentration was 3 mM glucose, 2 mM Gln, 6 mM lactate, 16 mM ammonia, 20 mM Ala, with an osmolality of 340 mOsm/l and a pH of 6.1 and without excessive accumulation of amino acids (< 5 mM for all amino acids).

Table 5-7: Enhancement of feeding strategies for infection of Sf-9 cells grown in fed-batch.

Feeding strategy used (quantity, supplement formulation and frequency of addition)					Total production (U β-gal / flask)	Yield (U β-gal / 10^6 cells)
0 hpi	24 hpi	48 hpi	72 hpi	96 hpi		
Positive control (total medium change-over at infection)					15680	48
Negative control (without medium change-over at infection)					850	3
20 % vol of supplement I	20 % vol of supplement V	20 % vol of $G_{50}Gln_{10}$ + $M_1P_{10}Y_{10}$	20 % vol of $G_{20}Gln_{10}$ + $M_1P_{10}Y_{10}$	20 % vol of $G_{20}Gln_{10}$ + $M_1P_{10}Y_{10}$	29760	102
20 % vol of supplement I	20 % vol of supplement V	20 % vol of $G_{50}Gln_{10}$ + M_1	20 % vol of $G_{20}Gln_{10}$ + M_1	20 % vol of $G_{20}Gln_{10}$ + M_1	34870	120
20 % vol of supplement I	20 % vol of supplement V	20 % vol of $G_{50}Gln_{10}$ + $P_{10}Y_{10}$	20 % vol of $G_{20}Gln_{10}$ + $P_{10}Y_{10}$	20 % vol of $G_{20}Gln_{10}$ + $P_{10}Y_{10}$	30960	107
20 % vol of supplement I	20 % vol of supplement V	20 % vol of $G_{50}Gln_{10}$	20 % vol of $G_{20}Gln_{10}$	20 % vol of $G_{20}Gln_{10}$	25920	89
20 % vol of supplement I	20 % vol of supplement V	None	None	None	15840	54

Supplement I : M_1G_{70} $_{20}P_{10}Y_{10}$; Supplement V : M_1G_{50} $_{10}P_{10}Y_{10}$

Fed-batches of Sf-9 cells were infected at 11.6 x 10^6 cells / ml, having been previously supplemented 24 hours before infection with an addition of 20 % vol of supplement I (M_1G_{70} $_{20}P_{10}Y_{10}$) at the density of 7.9 x10^6 cells/ml. After a single uniform addition of 20 % vol at 0 hpi of supplement I (M_1G_{70} $_{20}P_{10}Y_{10}$) and one of 20 % vol of supplement V (M_1G_{50} $_{10}P_{10}Y_{10}$), different supplement formulations were tested for the three last additions. Infections of batches at 9.0x10^6 cells/ml (with and without any supplementation) are reported in the Table for comparison (positive and negative controls).

For Sf-9, analogous observations on the hydrolysates' effects were made (Table 5-7). Glucose and Gln were necessary until the end of the infection. The accumulation of by-products for the best conditions (120 U β-gal/10^6 Sf-9 cells) stayed low (2 mM lactate, 3 mM ammonia, 16 mM alanine and low amino acid concentration < 3 mM).

5.4. DISCUSSION

5.4.1. GROWTH PHASE

In the screening experiments, we chose to test separately each compound that could accelerate the growth phase and extend the stationary phase of cells. As expected, glucose, Gln and Yeastolate have a positive effect on the growth phase of High-Five cells (Tables 5-1 and 5-2). The involvement of Gln in the energetic metabolism of insect cells is known to be useful to their growth (Rhiel et al. 1997). Yeastolate is composed of peptides, free amino acids (around 30 %), nucleic acids and carbon compounds and contains a rich source a vitamin B complex (Vaughn and Fan 1997). Its importance for insect cell growth and protein production has been observed by several authors (Bédard et al. 1997; Elias et al. 2000; Nguyen et al. 1993) due to its nutritional effects and especially its "growth promoting substances" (Wu et al. 1998). Its importance for fed-batch culture of Sf-9 cells in serum-free culture has already been reported (Nguyen et al. 1993).

The beneficial effect on prolonging the stationary phase was due to glucose and Primatone. The influence of Primatone on the extension of the stationary phase has been already noted on hybridoma cells (Schlaeger 1996b) and on insect cell (Ikonomou et al. 2001) and attributed to its antiapoptotic properties. The glucose concentration was fixed at 70 mM to prevent its excess (subsection 5.3.1.2.). The Gln concentration proposed was 20 mM thanks to its better effect on cell growth compared to 10 and 40 mM (subsection 5.3.1.3.). The concentrations of Primatone and Yeastolate were fixed at 10 g/l because the Yeastolate is known to inhibit the growth of Sf-9 at 16 g/l in medium (Drews et al. 1995). These compounds, except for Primatone, have already been used for the supplementation of fed-batch cultures of Sf-9 cells by several authors (Bédard et al. 1997; Chiou et al. 2000; Elias et al. 2000; Kim et al. 2000; Nguyen et al. 1993; Reuveny et al. 1991; Wu et al. 1998).

Trends regarding the separate effects of glucose, Gln, Yeastolate, and lipids have been reported for fed-batch cultures of High-Five insect cells (Wu et al. 1998). However, in our hands, these separate additions gave relatively poor results because the cells required more than one of these ingredients simultaneously. Thus, we decided to add the key supplemented ingredients by dissolving them in medium (Table 5-3).

For High-Five cells in growth phase, a single addition can increase cell density, whereas further additions are wasteful (Figure 5-1). The end of the growth of High-Five cells is not a consequence of a lack of nutrients but, rather, is caused by the increase of metabolic waste products and osmolality. If too many additions were made, the cells wasted nutrients and produced high levels of lactate and ammonia (data not shown) (Drugmand et al. 2005). This is in accordance with previous observations (Mendonça et al. 1999; Rhiel et al. 1997).

Concerning Sf-9 cells, multiple additions of nutrients allow to reach a high cell density (Figure 5-2). We obtained a cell density of $25x10^6$ cells/ml (with three pulses of $M_1G_{140}Gln_{40}P_{20}Y_{20}$) whereas Bédard et al. (1997) reached $20x10^6$ cells/ml with an amino acid and yeast extract supplement and Elias et al. (2000) obtained $52x10^6$ cells/ml with a complex supplement composed of glucose, amino acids, lipids, vitamins and yeast extract added continuously to prevent a steep increase of osmolality. In addition, we saw that adding a supplement with a strength factor of 2 gave unsatisfactory results.

5.4.2. Infection phase

We had initially hypothesized that these supplements optimised for growth would constitute a reasonable basis for good production, even though the metabolism of insect cells is known to be altered in infection compared to the growth phase (Rhiel et al. 1997). Thus, for infection, an adaptation of the feeding strategy seemed important, as summarized in the following questions: when ? how much ? how many times ? at what concentration ?

Both for High-Five and Sf-9, a single addition at the time of infection gave poor results because cells tend to waste nutrients and the excessive increase of osmolality can be toxic to the cells (Elias et al. 2000; Palomares and Ramirez 1998). Splitting the supplement in three fractions added every two days improved the production compared a single addition (subsection 5.3.2.1. and 5.3.2.2.). At the same overall quantity of nutrients, the cells produced less when fed a more concentrated supplement due to the accumulation of by-products and the increase in osmolality (Rhiel et al. 1997).

When we infected High-Five cells grown in fed-batch cultures, we obtained lower production than upon infection of simple batches (subsection 5.3.2.1.). The yield of production of the cells decreased with the accumulation of by-products, a tendency that is consistent with previous observations (Rhiel et al. 1997).

Concerning Sf-9 cells, their infection in fed-batch cultures gave similar production levels as the infection of batch-grown Sf-9 cells (Table 5-4 part B, Table 5-5). The product yield was better at the cell density of 12.8 than $15.6x10^6$ cells/ml, however the volumetric production was similar for the two infection densities as previously seen by Bédard et al.

(1997). Furthermore, workers in the same group infected Sf-9 cells at between 12 and $17x10^6$ cells/ml, obtaining a higher protein production at a cell density around $14x10^6$ cells/ml (Elias et al. 2000). Nevertheless these previous works that have attained higher cell densities than ours in the growth phase, have focussed mainly on the increase of the final cell density and not on the cell productivity. Their productions normalized on a per cell basis are between 1.5 and 2 fold lower than those reported in our present work. Yet, our results are not only due to the difference in feeding strategies but also to the difference of serum-free media (Ikonomou et al. 2001).

The adaptation of the added nutrients to the cells' requirements, which forms the basis for the strategies described in this paper, is crucial to prevent the waste of nutrients by the cells, and to decrease the residual accumulation of metabolites and by-products.

The best infection strategies found in this study for optimal protein production consisted of infections of High-Five cells previously grown in batches (at ca. $4\text{-}5x10^6$ cells/ml) and infections of Sf-9 cells previously grown in fed-batches (at ca. $11\text{-}13x10^6$ cells/ml), accompanied by various pulses of enriched medium (factor strength of 1, Tables 5-4, 5-5, 5-6 and 5-7, Figure 5-3). For High-Five cells, three pulses (of 20 % of the initial culture volume) were retained, while Sf-9 cells required five pulses (of 20 %) of a less concentrated supplement. The glucose and Gln concentrations in the pulses were gradually decreased during the infection as a function of the evolving instantaneous cell requirements. Approximately the same total quantity of glucose was added for both cell lines, at around 40-45 mmoles/l, even though the Sf-9 cell density at infection was three fold higher. This is to be expected, since High-Five cells consume more glucose than Sf-9 (Rhiel et al., 1997). We found that High-Five cells needed Gln only in the first two additions (total amount of 6 mmoles/l) whereas Sf-9 cells needed it throughout the infection phase (total amount of 14 mmoles/l).

For both cell lines, the supplement optimised for infection must contain Primatone and Yeastolate only in the first additions (Tables 5-6 and 5-7). The presence of these compounds seems to be required for their growth and antiapoptotic and growth promoting properties and not for their nutritional value. Amino acids, in contrast, are required throughout the infection (Donaldson and Shuler 1998). The use of complete medium during the whole infection phase sustains also the cells' needs for amino acids, lipids and vitamins during this period. Moreover, trace elements found in the medium such as zinc, iron and copper are also required by the cells.

5.5. CONCLUSION

In conclusion, the strategies described as a means to optimize protein production have as their target the decrease of the overall production cost with the IC-BEVS: there is neither a need for cell retention, nor one for a continuous perfusion system, plus lower medium quantities are required. To reach high protein production, energetic nutrients (glucose and Gln) must be added during the infection as a function of cellular needs, in the form of enriched medium (containing amino acids, vitamins and lipids) plus hydrolysates.

The medium must be added during the whole period of infection, whereas hydrolysates are used at the beginning of the infection. The best fed-batch productions titres obtained with High-Five cells infected with a baculovirus encoding β-gal and seAP were 5.5-fold higher compared to a batch control and, respectively, 30 % and 45 % higher compared to a total changeover of medium (Table 5-6). With Sf-9, the best production titres reached was, respectively, 16- and 2- fold higher compared to an infected batch control and a batch control with a total medium changeover before infection (Table 5-7).

Such strategies should constitute a good basis for future industrial applications that will rely on optimized High-Five and Sf-9 cells fed-batch cultures.

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6

PART III: INSECT CELL PROCESSES

Chapter 6 : Continuous insect cell cultivation in a packed-bed reactor for protein production using the Baculovirus Expression Vector System

6.1. INTRODUCTION

The Insect Cell-Baculovirus Expression Vector System (IC-BEVS) is a valuable platform for the production of recombinant proteins and viral pesticides (Agathos, 1994). This transient expression system is based on a co-culture of insect cells and baculovirus (Smith et al. 1983). Usually, in such a system, insect cells are cultivated in batch culture and are infected with the baculovirus when they have reached a sufficient cell density before the stationary phase. The productivity of a recombinant protein in the IC-BEVS is a function of the cell line, the virus construction (Possee 1997), the medium used (Schlaeger 1996), etc. The cell density at infection and the multiplicity of infection (MOI) (or number of virus per cell), the number of passages of the virus, the "culture age", the state of medium depletion are major factors affecting protein productivity (Chico and Jäger 2000; Ikonomou et al. 2003; Jäger 1996).

The use of high density culture is a challenge for the industrial exploitation of insect cells and various strategies have been proposed to increase cell density and recombinant protein production (Palomares et al. 2006). One such strategy is perfusion culture, which allows to reach a high cell density in a small volume (Ikonomou et al. 2003). Unfortunately, perfusion is difficult to control and the occurrence of high shear stress in retention systems such as hollow fibres can be deleterious to the cells (Ozturk 1996). On the other hand, microcarrier cultivation is relatively easy to apply, allowing the propagation of both adherent and non-adherent cells at large scale and increasing considerably the volumetric production. However, the anchorage-independent nature of most insect cell lines and the one-shot, lytic nature of the BEVS can cause various problems (Ikonomou et al. 2002). The turbulent flow due to high agitation and the formation of cell aggregate bridges between carriers can render the cultivation more heterogeneous (Drugmand et al. 2001).

Like other methods, packed-bed bioreactors have benefits and drawbacks. Their ability to retain the cells can result in high cell densities within small bioreactors under low shear stress (Reuveny 1990). Yet, problems include the difficulty to estimate cell density, the presence of heterogeneities inside the bed and the limited diffusion and transport of gases and metabolites inside the bed (Rundstadler et al. 1990). Moreover, the packed cells must remain viable and productive for prolonged culture duration (Meuwly et al. in press). However, these processes can bring new opportunities to the industrial application of insect cells. Usually fixed-bed reactors for animal cell culture contain macroporous supports compacted or non-woven fibres placed into a retention basket (Goosen 1993). Up to now,

the majority of insect cell immobilization studies have utilized microcarriers, fibrous packed-beds or encapsulation (Ikonomou et al. 2003), with only a few reports on insect cell cultivation in packed-bed bioreactors (Chiou et al. 1991; Chiou et al. 1998a; Chiou et al. 1998b; Kompier et al. 1991).

The choice of a carrier type must satisfy economic criteria (cost of the carrier, quantity required, etc.), and the physical and chemical properties of the carrier (chemical composition of the inner core and the surface of the carrier, porosity, roughness, etc.) must be adapted to the cultivation and production requirements (Lazar 1991). Related cultivation and production parameters include the seeding efficiency, the immobilisation efficiency (Hu and Dodge 1985), the cell growth rate, the volumetric and specific production of the immobilized cells (Kong et al. 1999) and the non-adhesion of the recombinant protein to the carrier (Reuveny 1990).

The goal of this work was to evaluate the feasibility of production a model recombinant protein by insect cells immobilized at high densities in a fixed-bed reactor. We tested two kinds of commercial PET macroporous supports (FibraCel® and BioNOC II™) and two kinds of non-woven fibres (glass and Dacron®) in agitated shake-flasks and in a conventional Celligen™ bioreactor equipped with a basket for the fixed-bed matrix.

6.2. MATERIALS AND METHODS

6.2.1. CELL LINES, VIRUS AND MEDIA

Trichoplusia ni High-Five™ and *Spodoptera frugiperda* Sf-9 insect cell lines were used. The High-Five cells were a gift of the Laboratory of Virology (University of Wageningen, The Netherlands), whereas Sf-9 were bought from Invitrogen (Carlsbad, CA). Suspension adapted cells were maintained in exponential phase in 125-ml shake-flasks (Corning, Acton, MA) at 27°C and 130 rpm with 25 ml of medium using inoculation densities of 0.2x106 High-Five cells/ml and 0.3x106 Sf-9 cells/ml, respectively. A recombinant *Autographa californica* nuclear polyhedrosis virus (AcMNPV) baculovirus encoding β-galactosidase (β-gal) was used (Invitrogen).

6.2.2. PREPARATION AND CHARACTERIZATION OF CARRIERS

Fibra-Cel® (New Brunswick Scientific, Edison, NJ) and BioNOC II™ (Cesco Bioengineering, Hsin-Chu, Taiwan) supports were prepared according to the manufacturer's instructions. Fibra-Cel are disks of 6 mm diameter and 600 μm thickness made of PET with a surface of 1200 cm^2/g dry weight. The Fibra-Cel disks have a grid of polystyrene to

rigidify the carriers (Website of New Brunswick Scientific: http://www.nbsc.com). BioNOC II are a new model of non-woven macroporous carriers in the form of a folded leaf developed for the growth of animal cells (Website of Cesco Bioengineering: http://www.cescobio.com). They are made of 100% pure PET with a treated surface area of 2000-2400 cm^2/g. Both carrier types have a density of 1.03-1.04 g/cm^3.

The non-woven Dacron® fibres (DuPont de Nemours, Paris, France) and ultrafine pure glass fibres (Vel: VWR International, Leuven, Belgium) were also tested. The Dacron fibres are non-treated soft filaments of 5 µm diameter of pure PET, whereas glass fibres have a diameter of 4 µm. The surface areas of Dacron and glass fibres are respectively estimated at 6000 cm^2/g and 4000 cm^2/g. Before use, the fibres were cleaned for 10 min with water/ethanol (30/70 v/v), for 2 h with 0.1 M citric acid, for 12 h with 0.1 M NaOH and were rinsed three times with PBS. Subsequently, they were placed in a siliconized container with Sigmacote (Sigma), autoclaved in PBS and rinsed twice with medium before inoculation.

6.2.3. Growth and infections

Cell growth was examined in YPR medium, a serum-free homemade medium previously developed in our laboratory (Ikonomou et al. 2001) to which dextran was added (1 g/L, MW:67 kDa, Sigma).

Experiments were performed in duplicate in 250-ml shake-flasks at 27°C containing 50 ml of medium and 10 g/L of supports at 130 rpm. The seeding densities were respectively 0.3 and $0.5x10^6$ cells/ml for Sf-9 and High-Five unless otherwise specified in the text. The inoculation was done in one third of the liquid volume and the flasks were slowly agitated every half hour for 3 hours. Supernatant samples were withdrawn daily (1 ml) to measure the density of free cells and the concentration of metabolites. Free cell density and viability were estimated by Trypan Blue dye exclusion (0.2% w/v final concentration). Supernatants were centrifuged (1000 x g, 10 min) before use for metabolite analysis. Samples consisting of 2 or 3 carriers or a few fibres were removed daily with a sterile forceps. The numbers of immobilized cells in the sample were estimated indirectly by biomass protein quantification using the Bradford assay (Bio-Rad Laboratories, Hercules, CA) after cell dissociation with Triton X-100 (1 % v/v in PBS, 2 h). The immobilized cell density was calculated by taking into account the totality of the cells in the sample and its mass (weighed after drying for 6 h at 70°C).

Infections were done in duplicate at a MOI of 2, when cells had reached saturation density, after a total medium change-over. The daily sampling of the culture led to a decrease in the total number of carriers or the quantity of fibres that was not proportional to

the decrease of the medium volume. Thus, we corrected the concentration of the extracellular and potentially trapped recombinant protein produced by the cells. For each sample, we took into account the protein that could have been produced by the immobilized cells that had been sampled at this point. This correction of intracellular protein was calculated as proposed by Ikonomou et al. (2002).

6.2.4. BIOREACTORS

Bioreactor cultivations were followed in a 2 L Celligen-Plus™ bioreactor of a 1.35 L working volume (New Brunswick Scientific) that was used with a fixed-bed basket of 500 ml containing 20, 35 or 50g of Dacron fibres, Fibra-Cel and BioNOC II carriers. The glass bioreactor was siliconized with Sigmacote (Sigma) to prevent cell adhesion. The bioreactor was inoculated with $0.2x10^6$ High-Five cells/ml or $0.3x10^6$ Sf-9 cells/ml (YPR medium, 27°C, dissolved oxygen (DO): 60% of air saturation, agitation: draft-tube impeller at between 80 and 150 rpm, pH control: 1 N NaOH). Two oxygen probes were used to measure the oxygen consumption of the cells, one below and one above the fixed bed. The DO was controlled based on the data from the upper probe. The dissolved CO_2 concentration in the media was estimated after calibration by measuring directly the pH in the sample and after flushing with nitrogen gas (15min, 2 L/min) to remove the dissolved CO_2. This methodology, based on various calibrations and the knowledge of the buffer strength of the medium, is possible with insect cell media that are buffered with a phosphate buffer, but not with other animal cell media buffered with a carbonate buffer. After calibration, we established the following equation (Equation 6-1) for YPR media where pH_i and pH_f denote the pH values before and after flushing, the dissolved CO_2 being expressed as a percentage of saturation.

$$pH_f = pH_i - (0.0197 \times pH_i - 0.110) \times [\% CO_2] \qquad \text{(Equation 6-1)}$$

A dynamic measure of oxygen consumption and CO_2 production rate by the cells was performed on each day of cultivation. During the cultivation, sampling of supernatant was done daily to count the free cells and to measure the concentrations of metabolites and recombinant protein. At the end of the cultivation, the reactor was opened aseptically and some samples of the fixed-bed were taken. The biomass in these samples was estimated *via* the Bradford protein assay.

6.2.5. BIOCHEMICAL ANALYSES

Concentrations of glucose, lactic acid and ammonia were assayed using a Bioprofile 100 Analyser (Nova Biomedical, Waltham, MA.). Glutamic acid (Glu) and glutamine (Gln) were determined with a Glu enzymatic kit (Boehringer, Mannheim, Germany). Other amino acids analyses were performed by high performance liquid chromatography (HPLC) according to Fekkes at al. (1995).

After calibration, the viability of the immobilized cell culture was estimated by measuring the lactate dehydrogenase (LDH) released by the dead cells in the supernatant using the Cytotoxicity Detection Kit LDH of Roche (Mannheim, Germany).

After thawing the samples, both the intra- and the extracellular β-gal produced were diluted in PBS buffer and were assayed according to Miller (1972).

The variation of pH due to the addition of base -or acid was investigated by potentiometric titrations of YPR medium with NaOH, HCl, L-lactic acid or NH_3. In YPR medium, we established the following empirical equation (Equation 6-2), in which each added amount is expressed in mmol/L, pH_i and pH_f being the pH before and after addition. (In other media, the buffer strength is different and the constants would change).

$$pH_f = pH_i + 0.0105 \times NaOH + 0.0107 \times NH_3 - 0.0127 \times \text{lactic acid} - 0.0130 \times HCl$$

(Equation 6-2)

Fluorescent microscopy using the Live/Dead cytotoxicity kit (Molecular Probes, Leiden, The Netherlands) served to evaluate cell viability on or inside the carriers. Confocal microscopy (Emission source: Bio-Rad MRC 1024, Bio-Rad, Hemel Hempstead, UK; Microscope: Zeiss Axiovert 135 M, Carl Zeiss AG, Göttigen, Germany) was employed to investigate the distribution of cells inside the carriers after fixation with formaldehyde (4% v/v in PBS) for 30 min and then stained with FM4-64 (Molecular Probes) and DAPI dyes before observation.

6.3. RESULTS

6.3.1. GROWTH STUDIES IN SHAKE-FLASKS

Growth of immobilized High-Five and Sf-9 cells was first evaluated in shake-flask culture in order to test the influence of the type of support, the cell lines and the media, on the seeding efficiency, nutrient consumption and recombinant protein production before testing the supports in packed-bed bioreactors. As the supports were different, we have chosen to express the cell density per amount of support.

Different seeding densities and durations were tested with High-Five and Sf-9 cells. The respective inoculation densities of $0.2x10^6$ and $0.3x10^6$cells/ml were found to be sufficient, in terms of lag phase length and growth rate, and were subsequently used for this study. Various periods of seeding (10 min, 30 min, 1 h, 2 h , 4 h, 6 h, 24 h) were tested. We calculated the seeding efficiency according to Equation 6-3, where X_i, V_i, X_f, and V_f are, respectively, initial and final free viable cell concentration (10^6 cells/ml) and volume (ml).

$$\text{Seeding efficiency} = \frac{X_i.V_i - X_f.V_f}{X_i.V_i} x100 \qquad \text{(Equation 6-3)}$$

The seeding was most efficient after 4 hours of inoculation (respectively, 88, 96, 70 and 60 % for Fibra-Cel, BioNOC II, Dacron II and glass fibres with High-Five cells). Shorter periods (less than 1 h) gave low seeding efficiencies, whereas much longer durations (above 6h) led to decreased growth rates. For both cell lines, the free cell density as determined on the day after the inoculation was less than 3 % of the total cell density.

After inoculation, the growth of the cells on the carriers could be modelled by a logistic equation (Equation 6-4), as previously used by Ikonomou et al. (2002), where X and X_0 are respectively the immobilized cell density (10^6 cells/g of support) at time *t* and at time *zero*, t is the time (h), μ is the specific growth rate (h^{-1}) and β is the inhibition constant (g support/10^6 cells). Integration of Equation 6-4 between time *zero* and time *t* gives Equation 6-5.

$$\frac{dX}{dt} = \mu.X.(1 - \beta.X) \qquad \text{(Equation 6-4)}$$

$$X = \frac{X_0.e^{\mu.t}}{1-\beta.X_0(1-e^{\mu.t})} \qquad \text{(Equation 6-5)}$$

The specific growth rate of the immobilized cells was lower than that of cells in suspension for both cell lines (Table 6-1). The growth rate was higher for High-Five than for Sf-9 cells for Fibra-Cel, Dacron and glass fibres, and similar for BioNOC II (Table 6-1, Figure 6-1). The maximal High-Five cell density was higher when grown on Dacron fibres (Figure 6-1). The Sf-9 cells reached the highest cell densities on Fibra-Cel and BioNOC II (Table 6-1). In all cases, when the medium was replaced to provide enough nutrients to the cells, their biomass increased slowly reaching saturation densities after 10 to 12 days of cultivation.

Densities of free High-Five cells during cultivation on Fibra-Cel and BioNOC II remained low during the cultivation (data not shown). Before confluence was reached, more than 95 % of the total High-Five cells were immobilized on the Fibra-Cel, BioNOC II and Dacron (Table 6-1). When the free Sf-9 cell density was higher than $1x10^6$ cells/ml, only the medium exchange maintained the free cell density below 25 % of the total cell density (Table 6-1, Figure 6-2).

Table 6-1: Cultivation of High-Five and Sf-9 cells on different carriers (10 g/L) in shake-flasks. Maximal immobilized cell densities (X_0) are the experimental values whereas specific growth rates (μ) and inhibition constants (β) are the kinetic parameters of the logistic equation (Equation 6-4) for each growth curve calculated by use of the Kaleidagraph curve fit utility (Synergy Software, Reading, PA).

Type of cells and carriers	Maximal immobilized cell density (10^6 cells /g support)	Immobilized cell density (% of total cell density)	Specific growth rate (h^{-1})	Inhibition constant (g support /10^6cells)
High-Five cells				
Fibra-Cel	250	96	0.0225	0.0039
BioNOC II	320	97	0.0195	0.0031
Dacron fibres	350	95	0.0250	0.0029
Glass fibres	135	82	0.0205	0.0071
Sf-9 cells				
Fibra-Cel	400	82	0.0190	0.0024
BioNOC II	360	84	0.0200	0.0026
Dacron fibres	280	75	0.0220	0.0031
Glass fibres	140	62	0.0170	0.0058

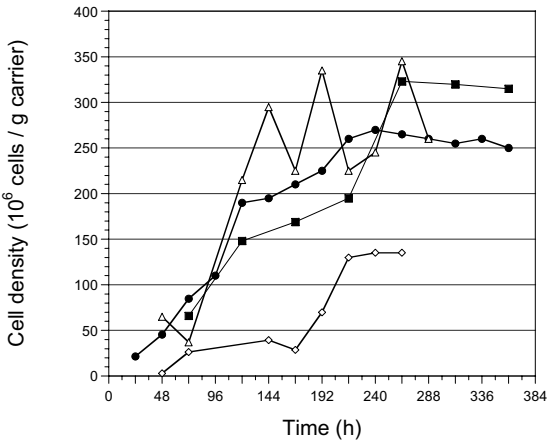

Figure 6-1: Effect of type of support on the immobilization efficiency of High-Five cells. Cells were immobilized cells on Fibra-Cel disks (●), BioNOC II supports (■), Dacron fibres (Δ) and glass fibres (◊) in shake-flasks at 10 g/L in YPR media. Time course of immobilized cell density. Medium was exchanged when the concentration of glucose decreased to 10 mM.

For both cell lines, the immobilized cell densities reached on glass fibres were low (Figure 6-1) and the majority of the cells grew free in the medium, even upon addition of serum in the medium or adsorption of collagen on the fibres (data not shown).

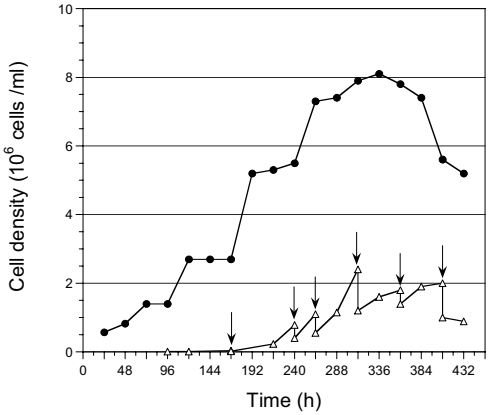

Figure 6-2: Cultivation of Sf-9 cells immobilized on BioNOC II (10 g/L, in YPR media). Time course of free (Δ) and immobilized (●) cells. The densities are reported to the volume of culture. Arrows indicate the change of the half of media.

Different media compositions (calcium-free, Pluronic-free, low osmolarity and serum-supplemented YPR media) were tested for the cultivation on Fibra-Cel (data not shown) and BioNOC II (Figure 6-3) supports. For both cell lines, the calcium-free formulation decreased the immobilized cell density (data not shown). Upon addition of serum to YPR medium (10 % v/v), the immobilized High-Five (Figure 6-3) and Sf-9 (data not shown) cell density reached were high but the presence of serum increased the free cell density (data not shown). For both cell lines, the low osmolarity media (260-270 mOsm/kg) gave high immobilized cell densities, but resulted in decreased cell viability. When High-Five cells were cultivated in Pluronic-free media, few free cells were present in the supernatant (below the detection limit of $1x10^4$ cells/ml). For Sf-9 cells, the absence of Pluronic F-68 did not diminish the free cell density but decreased the viability of this cell line, as well as its maximal cell density (data not shown). The highest cell densities were obtained in normal YPR medium for Sf-9 cells (data not shown) and in Pluronic-free YPR for High-Five cells (Figure 6-3).

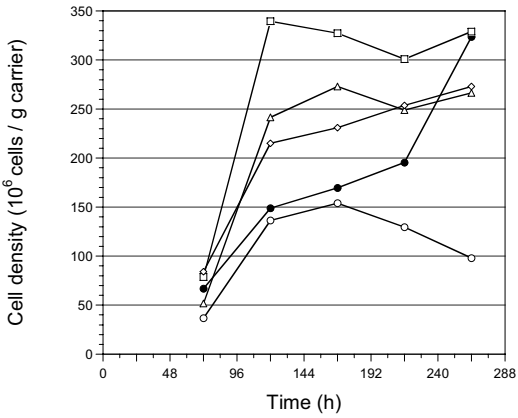

Figure 6-3: Effect of medium composition on the immobilization of High-Five cells on BioNOC II supports. Time course of High-Five immobilized cells on BioNOC II (10 g/L) in "standard" YPR medium (●), in Pluronic-free YPR (□), in calcium-free YPR (○), in YPR with 10 % serum (Δ) and in YPR with a lower osmolarity (260-270 mOsm/kg instead 350-360 for YPR) (◊). The medium was changed when glucose decreased to 10 mM.

Microscopic observations of the Fibra-Cel, BioNOC II and Dacron with a Live/Dead test indicated that the viability of Sf-9 and High-Five cells on the support were very high (> 95 %), without any necrotic core inside the carriers. In serum-free media, confocal microscopy indicated that the High-Five and Sf-9 cells grew in aggregates on the crossings of fibres inside the carriers. Whereas in the presence of serum, cells grew homogenously on the fibres as shown by Kompier et al. (1991) and Ikonomou et al. (2002), confocal microscopy observations of carriers showed heterogeneity inside the Fibra-Cel disks: there were fewer cells inside the carriers than on the edges, almost 70 % of the cells being trapped within the first 80 µm of depth. With BioNOC II supports, the cell distribution was more homogeneous inside the carriers except in the folding of the carriers where there were very few cells. With Dacron and glass fibres, the cells grew isolated along the fibres.

When cultivated on Fibra-Cel, BioNOC II and Dacron, Sf-9 and High-Five cells displayed a relatively constant nutrient consumption rate. The volumetric metabolic rates increased during the cultivation and became constant when cells reached confluence (Figures 6-1 and 6-4). The specific consumption rate of glucose (ca. 420 µmol/h.10^9 High-Five cells and ca. 370 µmol/h.10^9 Sf-9 cells) was similar when cells were immobilized on BioNOC II, Fibra-Cel and Dacron fibres (data not shown). However, the lactate production rates of immobilized cells differed from one carrier to another one: High-Five cells produced high amounts of lactate when cultivated on Dacron fibres (ca. 220 µmol/h.10^9cells) and less when cultivated on Fibra-Cel or BioNOC II carriers

(between 40 and 70 µmol/h.10^9 cells), whereas Sf-9 cells did not produce lactate (data not shown). The production of ammonia by High-Five and Sf-9 cells cultivated on Fibra-Cel, BioNOC II or Dacron was constant, amounting, respectively to around 140-220 and 80-100µmol/h.10^9cells.

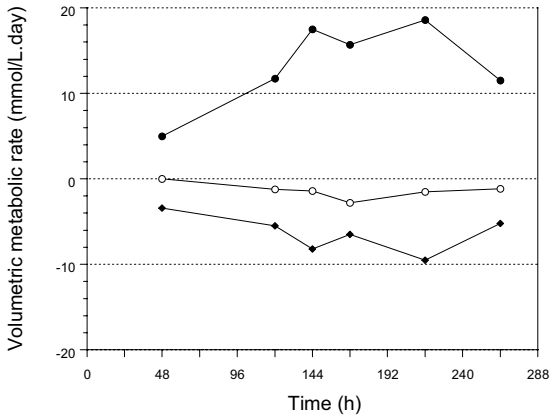

Figure 6-4: Time course of nutrient consumption and by-product formation by High-Five cells immobilized on Fibra-Cel carriers in shake-flasks (10 g support/L). Volumetric glucose (●) consumption rate, and lactate (○) and ammonia (♦) production rate. The corresponding cell densities of this experiment are given in Figure 6-1. Medium was exchanged when the residual concentration of glucose reached 10 mM.

6.3.2. INFECTION STUDIES IN SHAKE-FLASKS

The effect of cell immobilization on recombinant protein production was investigated by infecting cells growing on carriers in shake-flasks, by a baculovirus expressing β-gal (Table 6-2). At a density corresponding to ca. 4.10^6 High-Five cells/ml or 5.10^6 Sf-9 cells/ml, cultures were infected, except on glass carriers, since such cell densities were never reached. For both cell lines, the production of β-gal by the immobilized cells was higher and prolonged, as compared to cells infected in suspension (Table 6-2). During the infection of immobilized cells, 75-80 % of the totality of the β-gal was extracellular at any given time (data not shown), whereas in suspension there was more intracellular β-gal at the beginning of the infection (0 to 72 h.p.i.), but almost all the recombinant protein (90 %) was extracellular at the end of the infection (120-144 h.p.i.). In the shake-flasks containing immobilized cells, the intracellular β-gal in the free cell fraction was very low (2-3 % of the total β-gal), due to the correspondingly low free cell density (data not shown).

Table 6-2: Volumetric production of β-gal by High-Five and Sf-9 cells grown on different solid supports in 125-ml shake- flasks (130 rpm; 27 °C; MOI: 2).

Type of cultivation	Total cell density (10^6 cells/ml)	Volumetric production of β-gal (U /ml)	Time of harvest (h.p.i.)
High-Five cells			
Fibra-Cel (10 g/L)	4.2	1115	240
BioNOC II (10 g/L)	4.1	1235	240
Dacron fibres (10 g/L)	4.2	1125	240
Glass fibres (10 g/L)	2.5	850	192
Suspension cultivation	4.0	850	144
Sf-9 cells			
Fibra-Cel (10 g/L)	5.3	965	192
BioNOC II (10 g/L)	5.2	1020	192
Dacron fibres (10 g/L)	5.3	980	192
Glass fibres (10 g/L)	3.5	610	192
Suspension cultivation	5.5	560	144

All infections were performed with a MOI of 2, after a total changeover of medium before infection (centrifugation for 10 min at 500xg for suspension cultivation). The total cell density was measured after the changeover of medium. For each cultivation, the infections were performed at the same density (of ca. 4.10^6 High-Five and ca. 5.10^6 Sf-9 cells/ml), except for glass fibres for which such densities were not reached. Time of harvest (h.p.i.) is the time when the recombinant protein concentration was maximal.

6.3.3. BIOREACTOR STUDIES

6.3.3.1. Growth studies of packed-bed of Dacron fibres

We cultivated High-Five cells on Dacron fibres in a packed-bed bioreactor at the concentrations of 0.040, 0.067 and 0.100 g Dacron per ml of bed in YPR-Pluronic-free medium. The time course of the concentration and volumetric consumption/production rate of glucose, oxygen, lactate and ammonia were followed.

During the first 5 days of cultivation at 0.067 g Dacron/ml of packed-bed, the reactor was maintained in a batch mode before beginning the medium perfusion (Figures 6-5 and 6-6). The perfusion rate was adapted so as to stabilize the glucose concentration at 20 mM (corresponding to one-third of the glucose concentration in the fresh medium). From day 2 to 7, the pH was controlled by point addition of 1 N NaOH, whereas after day 7, the feeding of the base was continuous. From the inoculation to 96 h, the cells consumed a lot of glucose and produced little lactate (Figure 6-5). But after 120 h of cultivation, the cells produced more lactate (1.2 mole of lactate produced per mole of glucose consumed) and more CO_2 (2-3 moles CO_2 / mole glucose), and consumed less oxygen (2.7 moles O_2 / mole glucose). The dissolved CO_2 accumulated in medium reached up to 22 % of saturation.

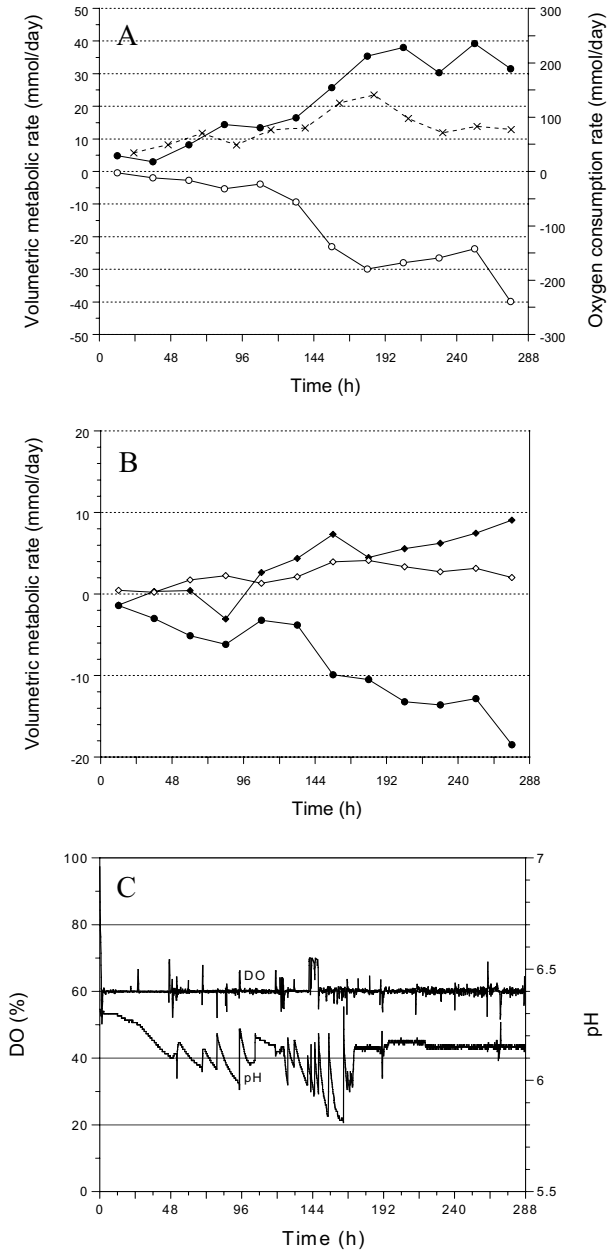

Figure 6-5: Cultivation of High-Five cells immobilized on Dacron fibres in a Celligen-Plus packed-bed bioreactor at 0.067 g Dacron / ml packed-bed. Time course of DO (panel C), pH (panel C), volumetric rate of consumption of glucose (panel A,●), oxygen (panel A, x), glutamine (panel B,◊) and glutamate (panel B,♦) and of production of lactate (panel A,○) and ammonia (panel B,■).

The pH drastically diminished (Figure 6-5), requiring a significant addition of base to stabilize it (62 mmoles/day during the last four days of cultivation). During cultivation, the cells consumed high amounts of Gln, Glu, (Figure 6-5) Asn (11 mmol/day at 240 h) and Asp (4 mmol/day at 240 h, data not shown) and produced a lot of ammonia (Figure 6-5). During the last four days of cultivation, the DO measured below the fixed-bed was between 8 and 12 % lower than that above the matrix (data not shown). The release of LDH indicated a cell viability around 92-93 % at the end of the cultivation (data not shown). Microscopic observations of samples indicated a homogenous distribution of the cells inside each sample.

At the end of the culture, the reactor was opened and samples of the bed matrix were taken. Vertical macro-heterogeneities in immobilized cell density were found from the top to the bottom, but no radial heterogeneities (Figure 6-6). By comparing the volumetric glucose consumption rate and the lactic acid production rate found in this experiment to the specific rates found in the shake-flask experiment, the cell density in the Dacron packed reactor could be estimated at between 130 and 160x10^{6}cells/g Dacron, whereas the average density measured *via* the Bradford protein assay was 147x10^{6}cells/g (Figure 6-6).

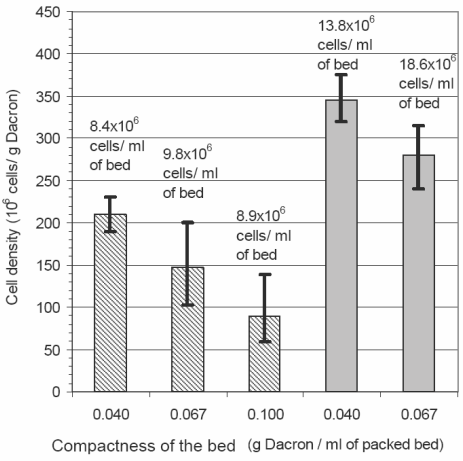

Figure 6-6: Cultivation of High-Five cells (dashed histograms) and Sf-9 (solid grey histograms) immobilized on Dacron fibres in Celligen-Plus packed-bed bioreactor at various levels of compactness. Cell densities (histograms) are average densities in the bioreactor expressed per gram of support estimated at the end of cultivation by the Bradford protein assay. Values indicated above the histograms are average densities expressed per volume of packed-bed. Bars represent the heterogeneity of the bed by indicating the difference between the cell density at the upper and lower part of the packed-bed (8 measurements for each part).

In another, similar experiment, with a smaller compactness of 0.040 g Dacron/ml of bed, the cell density reached at confluence was higher and more homogeneous (Figure 6-6). The average cell density estimated on the basis of metabolite production or consumption rates was 200-230x10^6cells/g vs. 212x10^6cells/g measured on the basis of the Bradford assay.

In another packed-bed, with a higher compactness of 0.100 g Dacron / ml of bed, the heterogeneity was more pronounced (Figure 6-6). After 6 days of cultivation in such a compact fixed-bed bioreactor, the concentrations of dissolved CO_2 (27 %) and lactate (38 mM) reached very high levels, whereas the pH was very difficult to control around 6.2-6.3 even if base was added and the agitation speed was increased from 80 to 170 rpm (data not shown). The cell viability estimated by the LDH assay was low (84 %, data not shown) and the cell density (measured by the Bradford assay) reached only 89x10^6 cells/g Dacron (between 80 and 100x10^6 cells/g estimated on the basis of metabolite rates). Moreover, the release of free cells was more extensive than with 0.067 g/ml of bed [7 % (with 0.100 g/l) vs. 4 % (with 0.067 g/l) of the total cell density].

Similar experiments were carried out using Sf-9 cells. When cultivated at the same degree of compactness, the Sf-9 cells reached higher cell densities than High-Five cells (Figure 6-6). Volumetric consumption of glucose of Sf-9 cells was quite similar to that of High-Five cells (data not shown) but Sf-9 cells had a lower rate of production of lactate (0.3-0.4 mmol /L.day). In these Sf-9 packed-bed cultivations, the dissolved CO_2 reached a lower level (7 %) than in the High-Five cell culture and the amount of base added was lower (12 mmol/day, data not shown). Unfortunately, the percentage of free cells in suspension was very high (18 % of total cell density, data not shown).

Due to the high heterogeneities observed in these Dacron packed-beds, we chose, instead, to test Fibra-Cel and BioNOC II carriers for infection studies.

6.3.3.2. Growth and infection studies of packed-beds of Fibra-Cel and BioNOC II carriers

Infections of High-Five cells immobilized on Fibra-Cel and BioNOC II carriers were performed in a packed-bed Celligen-Plus bioreactor at 0.067 and 0.040 g support / ml of bed using Pluronic-free medium.

High-Five Cells were cultivated on BioNOC II carriers at 0.040 g support / ml of bed (Figures 6-7, 6-8 and 6-9) for 6 days in batch mode before starting perfusion. At day 11, the perfused reactor was infected and the perfusion rate adapted to keep the glucose concentration below 20 mM. During the infection, the perfusion rate was adapted

to prevent the dilution of r-protein and accumulation of by-products. The pH was controlled at above 6.1 with continuous addition of 1 N NaOH (Figure 6-8). The volumetric metabolite rates increased during the period of cultivation until 168 h, and then were stabilized when the cell density reached saturation (Figure 6-7). During the growth phase, the volumetric consumption rate of glucose was almost equal to the volumetric production rate of lactate (Figure 6-7). Moreover, cells consumed low amounts of oxygen (between 3.5 and 4.5 moles of O_2 consumed per mole glucose produced, Figure 6-7), producing high amounts of CO_2 (Figure 6-8) and of ammonia (Figure 6-7).

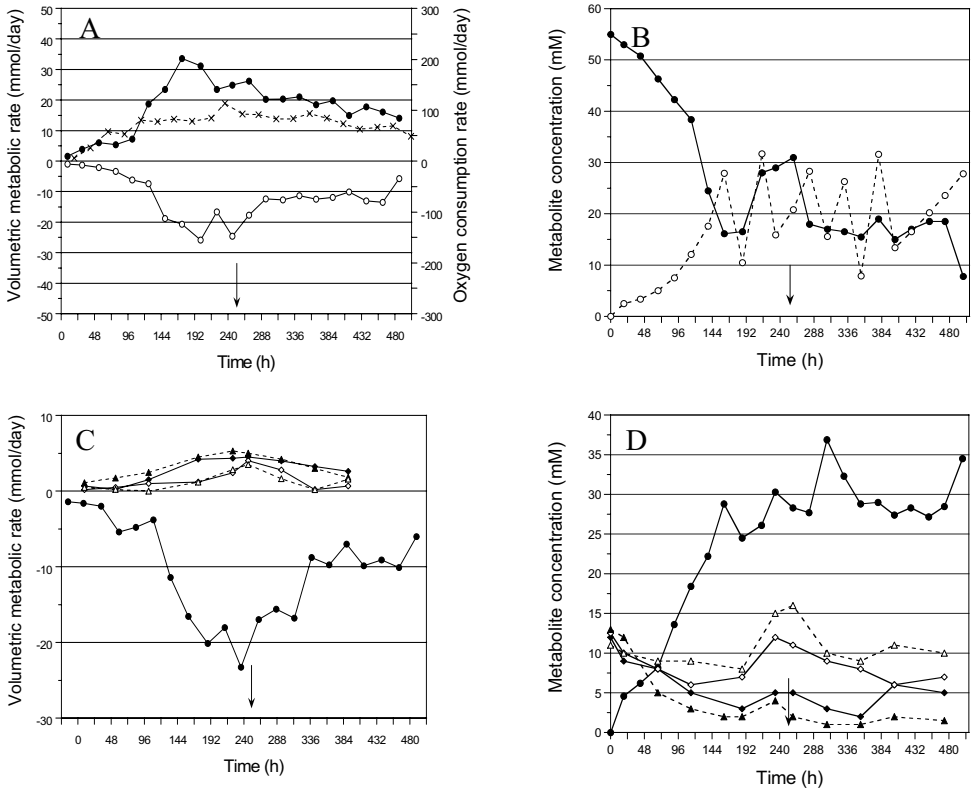

Figure 6-7: Cultivation and infection of High-Five cells immobilized on BioNOC II carriers in a Celligen-Plus packed-bed bioreactor at 0.040 g BioNOC II/ml of bed. Time course of the nutrients concentration and volumetric production/consumption rate: glucose (panels A and B,●), oxygen (panel A, x), lactate (panels A and B, ○), ammonia (panels C and D,●), glutamine (panels C and D, ◊), glutamate (panels C and D,♦), asparagine (panels C and D,Δ) and aspartate (panels C and D,▲). Arrows indicate the time of the infection.

Contrary to the Dacron packed-beds, the DO values measured by the oxygen probes above and below the bed were similar (data not shown) and the dissolved CO_2 accumulated was lower, Figure 6-8). Just before infection (240 h), the cell density was estimated (based on metabolite consumption) at 410±15.10^6 High-Five cells/g of BioNOC II (Table 6-3). The viability was estimated to be higher than 95 % (based on the release of LDH). During infection, the consumption rate of glucose and oxygen and the production of lactate and ammonia slowly diminished (Figure 6-7), while the addition of base was relatively constant (ca. 30 mmoles/day, Figure 6-8).

The concentration of β-gal in the medium was related to the specific production rate and the perfusion rate (Figure 6-9). During the first 144 h.p.i., the r-protein concentration increased up to ~800 U β-gal/ml, while afterwards r-protein titre continued to increase but its concentration remained stable due to medium perfusion. At the end of the infection phase (240 h.p.i.), the total production titre was 3.1x10^6 U β-gal, i.e. 2.5 fold higher than for the batch control experiment.

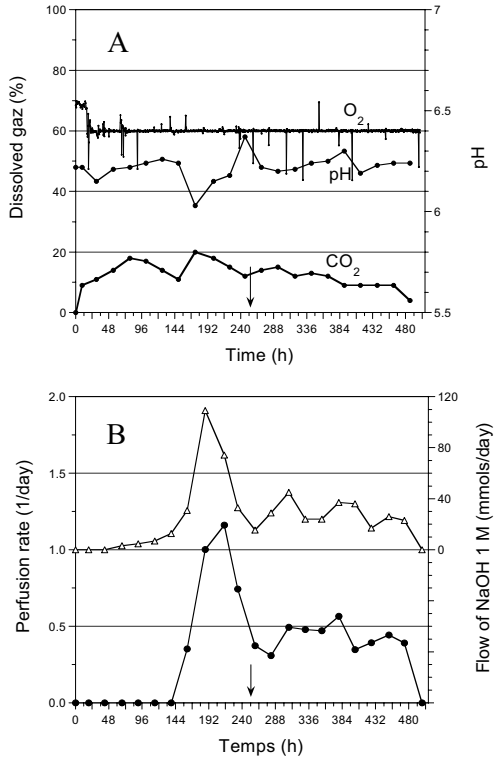

Figure 6-8: Cultivation and infection of High-Five cells immobilized on BioNOC II carriers in a Celligen-Plus packed-bed bioreactor at 0.040 g BioNOC II/ml of bed. Time course of pH, dissolved oxygen, dissolved carbon dioxide (panel A), perfusion rate (panel B,●) and base flow (panel B,Δ). Arrows indicate the time of the infection.

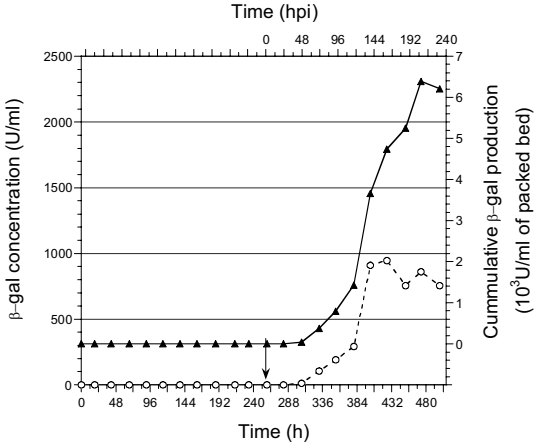

Figure 6-9: Cultivation and infection of immobilized High-Five cells on BioNOC II carriers in a Celligen-Plus packed-bed bioreactor at 0.040 g BioNOC II/ml of bed. Time course of β-gal concentration (○, U/ml) and β-gal cumulative volumetric production (▲) expressed in units of β-gal produced by volume of fixed-bed. Arrow indicates the time of the infection.

Table 6-3: Cultivation of High-Five cells immobilized on Fibra-Cel and BioNOC II carriers in a Celligen-Plus packed-bed bioreactor. Perfusion rate was controlled to keep the glucose concentration at around 20 mM. Cell density was estimated on the basis of volumetric metabolite rates (as explained in Materials and Methods)

Carrier	Compactness of bed (g support / ml of bed)	Glucose concentration in fresh medium (mM)	Density before infection (10^6 cells/ g support)	Density before infection (10^7 cells / ml of fixed bed)	Total of β-gal production (10^6 U/ run)	Final β-gal concentration (U/ml)	Time of harvest (h.p.i.)	Perfusion rate during infection (1/day)
Fibra-Cel	0.040	55	380 ± 25	~1.5	2.85	820	240	~ 0.45
BioNOC II	0.040	55	410 ± 15	~1.7	3.10	800	240	~ 0.50
	0.067	55	370 ± 20	~2.4	2.40	750	288	~ 0.65
	0.040	33	390 ± 20	~1.6	2.50	420	240	~ 0.85

In an experiment using the same conditions (Celligen-Plus, YPR-Pluronic free medium, 0.040 g support / ml of bed) but with Fibra-Cel supports, the biomass estimated before infection and the production titre of β-gal were slightly lower than with BioNOC II carriers (380 vs. 410x10^6 cells/g and 2.85 vs. 3.10x10^6 U β-gal/run, Table 6-3), as shown in shake-flask studies (Tables 6-1 and 6-2).

At the end of these cultivations, the reactors were opened and samples of bed were taken. For both carriers, the sampling of the beds showed neither horizontal, nor vertical heterogeneity inside the matrices.

In another packed-bed experiment with a more compact bed (0.067 g BioNOC II/ml of bed), the estimated biomass per gram of support, before infection, was lower compared to that at a compactness of 0.040 g/ml of bed. However, the total biomass per volume of packed-bed was compensated by the increase of the amount of support (Table 6-3). Although there were more cells in total, the production titre of β-gal was lower than at a compactness of 0.040 g /ml (Table 6-3).

In order to investigate the effect of medium composition on r-protein production in the packed-bed reactor, an experiment with High-Five cells cultivated on BioNOC II supports at 0.040 g / ml of bed but in YPR medium with lower glucose concentration (33 mM instead of 55 mM) was carried out. Cells had similar growth and metabolism rates (data not shown) as in the cultivation with 55 mM of glucose. Moreover, at the time of cell confluence, the cell density was similar (Table 6-3). However, to maintain the level of glucose as before (above 20 mM), perfusion rate had to be ca. 1.6 fold higher (Table 3) than in the run with 55 mM of glucose, the total production was lower (2.5 vs.3.1x10^3 U β-gal/run) and the titre more diluted (420 vs. 800 U β-gal/ml) (Table 6-3).

6.4. DISCUSSION

Since the first report of insect cell cultivation in a fixed-bed reactor by Kompier et al. (1991), the use of macroporous carriers in animal cell culture for the production of biologicals has become quite common. The cultivation of anchorage independent insect cell lines (like Sf-9 and High-Five) was shown to require carriers able to fix the cells on the support, but also to trap them (Ikonomou et al. 2003).

Inoculation and medium required

In the present work, the presence of serum increased the seeding efficiency and the growth rate during the first day of cultivation (Figure 6-1), consistent with a decrease in the release of cells from the fixed-bed (Barnes 1984). The use of low osmolarity or calcium-free medium appeared deleterious for the cultivation of High-Five (Figure 6-3) and Sf-9 cells on carriers, in line with work by Miller et al. (2000) who have shown a divalent cation-dependence of the adhesion of *Drosophila* cells. When cultivated in Pluronic-free YPR medium, the growth rate of High-Five cells was higher due to a drop in the release of cells in the medium (Figure 6-3). Typically, Pluronic F-68 protects suspension-growth from adverse effects of agitation and sparging (Murhammer and Goochee 1988) but has no effect

on immobilized cells (Miller et al. 2000). However, its absence could cause a decrease of viability in free cells, as seen with Sf-9 cells (Table 6-1, Figure 6-2).

The seeding efficiency was a function of the support type, the medium, the cell line and the duration of the process of seeding (Ikonomou et al. 2002). For both cell lines, the best seeding time was 4 h. Glass fibres had a poor ability to trap the cells, hence they are not a good candidate for use in a fixed-bed bioreactor (Figure 6-1). Conversely, the shake-flask studies showed the other supports are promising for use in fixed-bed configurations (Figure 6-1, Tables 6-1 and 6-2). Upon inoculation at a standard density (i.e. $0.3x10^6$ and $0.5x10^6$ cells/ml, for High-Five and Sf-9 respectively), saturation was reached between 200 to 300 h, as a function of the carrier used (Figures 6-1, 6-2 and 6-3). The growth rate of both cell lines cultivated on BioNOC II, Fibra-Cel or Dacron fibres were found to be near the μ_{max} for these two cell lines (Ikonomou et al. 2002).

Estimation of biomass in the packed-bed

During cell cultivation in a fixed-bed bioreactor, the major difficulty is the estimation of immobilized cell density (Rundstadler et al. 1990). Methods able to directly measure the cell density (crystal violet or protein biomass estimation) require direct sampling of the bed. The crystal violet method allows to directly estimate the density of cells immobilized on the support by counting cell nuclei after cell lysis, but seems to underestimate cell density when compared to protein biomass measurement (Berry et al. 1996). Thus, the Bradford protein assay which measures the protein content of the cell biomass seems to be a better choice.

On the other hand, indirect estimation of the cell density on the basis of metabolite consumption rates is difficult, because the specific consumption rates are often not constant during the cultivation and because the metabolism of adhering cells can differ from that of suspension cells (Ikonomou et al. 2002). For example, production of lactate was higher for cells in the packed-bed as compared to those in suspension and consumption rate of glucose was between 2 and 2.5 fold higher than for cells in suspension. On, the other hand, we have shown, in preliminary studies in shake-flasks, that Sf-9 and High-Five cells cultivated on Fibra-Cel, BioNOC II or Dacron, consumed nutrients at a relatively constant specific rate (see Results).

Hence, the use of such preliminary studies in shake-flasks, where sampling is convenient, to investigate the growth (by direct measurements) and the specific production/consumption rate of cells, is an efficient methodology for calibrating the parameters required for biomass estimation. After such a calibration in shake-flask, the

evaluation of immobilized High-Five density in bioreactor, based on glucose consumption and lactate production rate, in the Dacron, Fibra-Cel and BioNOC II bioreactors, was possible. Our cell density estimations, gave comparable values to those from a direct biomass measurement (see Results).

Concerning the viability, the low release of LDH during the cultivation and the microscopic observations indicated that the majority of the cells present in the fixed bed were alive, whereas dead cells were detached from the carriers.

Micro- and macro-heterogeneities in the packed-bed

Packed-bed studies with High-Five cells revealed heterogeneities in the bed (Figure 6-6). At a compactness of 0.040 g support/ml of bed, the macro-heterogeneity was higher when the fixed-bed was made up of Dacron fibres (Figure 6-6), as compared to macroporous supports such as Fibra-Cel or BioNOC II, whereas there was no significant difference between the total void volume of the Dacron fibres, Fibra-Cel or BioNOC II fixed-bed (*e.g.* at 0.040 g support/ml of bed: 96 % void). Nevertheless, there was more void inside the carriers in the case of BioNOC II (76 %) and more void between carriers in the case of Fibra-Cel (93 %).

When High-Five cells were cultivated in a Dacron fibre fixed-bed reactor, a cell density gradient was observed in the direction of the liquid circulation in the bed (Figure 6-6). A clear concentration gradient across the packed-bed was also measured in the case of oxygen, but not for other metabolites (glucose, lactate, pH and pCO_2), similar to observations on CHO cells cultivated on Fibra-Cel (Meuwly et al. in press). Oxygen tends to be the most crucial substrate in terms of gradient due to its poor solubility in the culture medium (Fassnacht and Pörtner 1999) and its high consumption by insect cells (Drugmand et al. in press-b). Macro-heterogeneities seem to be absent in Fibra-Cel and BioNOC II beds (macroporous carriers) where no oxygen nor metabolite gradients have been observed.

Nevertheless, microscopic observations of Fibra-Cel and BioNOC II have shown a high micro-heterogeneity in the cell distribution inside the carriers (Drugmand et al. in press-a; Ikonomou et al. 2002), while with the Dacron bed the cells grew along the fibres with practically no micro-heterogeneity. In the case of fibres, cells are probably growing along the critical paths in the bed (Lazar 1991; Tokashiki 1991).

Hence, the macro-heterogeneity, present in a packed-bed made up of compacted fibres seems to affect mainly the liquid circulation inside the bed, DO and pH. On the other hand, the micro-heterogeneity which is a function of the void volume inside the macroporous supports, did not affect liquid circulation inside the bed. Given that all fixed-

beds display gradients and heterogeneities (Lazar 1991), the goal of fixed-bed culture is to diminish their negative effects to the maximum. Thus, the compactness of the fixed-bed is an important parameter, to prevent excessive heterogeneities and gradients. According to our own observations (subsection 6.3.3.1 and 6.3.3.2.), a compactness of ca. 0.040 g support/ml of bed of non-woven Dacron fibres (Figure 6-6) was the limit for the cultivation of High-Five cells, and ca. 0.067 g support/ml of bed in the case of macroporous supports (Fibra-Cel or BioNOC II, Table 6-3). These limits are probably the same for other types of support, and for other insect cell lines, such as Sf-9 cells, cultivated in a conventional reactor equipped with a fixed-bed matrix.

Infection of insect cells cultivated in packed-bed

For both cells lines, the recombinant protein production by the immobilized cells reached comparable values except for the glass fibres for which the production was lower due to the lower cell density (Table 6-1). The time of harvest increased (Table 6-1) due to the non-synchronous infection of cells on supports, as previously noted (Ikonomou et al. 2002). The protein titre attained by immobilized cells on supports was higher than in batch (Table 6-2). Nevertheless, the time of harvest was longer than in suspension. Tested in a bioreactor context, the production of High-Five cells infected on Fibra-Cel carriers appeared quite similar to that with BioNOC II supports (Table 6-3). When the compactness of the fixed-bed increased, the specific cell production slowly decreased and the time of harvest increased (Table 6-3). During the infection, the perfusion rate must be as low as possible to prevent the dilution of the recombinant protein produced. Thus, in the infection phase, a perfusion rate lower than 1 volume/day with a medium of high nutrients content (e.g. glucose > 40 mM) is desirable if the accumulation of by-products is not too high.

Perfusion rate and glucose concentration

During the growth of insect cells in a packed-bed bioreactor, the perfusion rate must be adapted to prevent the accumulation of by-products. When a medium with 55 mM of glucose was used, the accumulation of by-products by High-Five cells (Figure 6-7) was higher than the estimated critical value: 25 mM of lactate and 30 mM of ammonia (Clausen et al. 1996) whereas the use of the same medium but with 33 mM of glucose did not lead to such by-product build-up, but required a higher perfusion rate (Table 6-3). Using a medium with still lower glucose concentration (below 25 mM) could not support high cell densities and may lead to wasting too much medium. Thus, the use of medium containing 25-50 mM glucose seems more desirable for cultivation of insect cells in fixed-beds. For the scale up

of insect cells in a fixed-bed reactor, the estimation of the by-products volumetric production rate must constitute a critical point, allowing to set the glucose concentration and the perfusion rate of the reactor.

pH control and base addition

During the packed-bed process studied here, High-Five cells produced more lactic acid and CO_2 than in suspension, and thus the pH had to be controlled by the addition of base. The use of 1 M NaOH instead of 0.1 M seems preferable, in order to prevent the dilution of the medium. The level of CO_2 accumulation acceptable for insect cells was estimated around 15 %.

Knowledge of the effect of dissolved CO_2 on the pH of YPR medium (Equation 6-1) and its buffer strength (Equation 6-2) could be used to establish the following equation (Equation 6-6), describing the effect of the modification of pH of YPR medium due to the addition of lactate, HCl, CO_2, ammonia and NaOH, where pH_i and pH_f are the initial and final pH, the lactate, ammonia, NaOH and HCL are expressed in mmol/L and the dissolved CO_2 as percentage of saturation:

$$pH_f = pH_i - (0.0197 \times pH_i - 0.110) \times [\%\ CO_2] \quad \text{(Equation 6-1)}$$

$$pH_f = pH_i + 0.0105 \times NaOH + 0.0107 \times NH_3 - 0.0127 \times \text{lactic acid} - 0.0130 \times HCl \quad \text{(Equation 6-2)}$$

$$pH_f = pH_i - 0.0105 \times NaOH + 0.0107 \times NH_3 - 0.0127 \times \text{lactic acid} - 0.0130 \times HCl - (0.0197 \times pH_i - 0.110) \times [\%\ CO_2] \quad \text{(Equation 6-6)}$$

Coefficients of these empirical Equations (6-1, 6-2 and 6-6) come from potentiometric titrations of YPR medium with HCl, NaOH, L-lactic acid and NH_3 (Equation 6-2), and from calibrations of CO_2 flushing in YPR medium (Equation 6-1) (see Materials and Methods for more details). Equation 6-6 is valid in the range of classic insect cell cultivation (pH: 5.8 to 7.2; ammonia and lactate: 0 to 30 mM; pCO_2: 0 to 20 %). In other insect cell media, buffer strength is somewhat different and constants would change slightly.

Therefore, knowledge of the effect of ammonia, lactate and CO_2 accumulation on the modification of pH (Equation 6-6), correlated with the measurements of their production rate could be used to predict the pH variation in an insect cell cultivation, and to monitor the pH control by addition of CO_2, acid or base.

In perfusion cultivation, the pH controlled by addition of acid or base is directly proportional to the pH of the fresh medium, the pH in the reactor, the perfusion rate and the production rate of ammonia, lactate and CO_2. Indeed, an increase of the perfusion rate results in an increase of base addition (Figure 6-8). Hence, in perfusion mode, the prediction of pH requires: the measurements of the production rates of ammonia, CO_2 and lactate; the rate of addition of acid (HCl) and base (NaOH); the use of Equation 6-6 and taking account of the renewal of medium by addition of a complete mass balance of H^+ and OH^- (function of the perfusion rate and the H^+ and OH^- concentrations in fresh medium)

Key parameters of insect cell cultivation in packed-bed

Our experimental data (subsection 6.3.3.) and our technical background indicate that the control of a packed-bed process of insect cells should take into account the following parameters (within their acceptable ranges) by increasing order of importance: the amino acid consumption rate, the DO (50-60 %), the pCO_2 (max 15-20 %), the perfusion rate (0.8-2 day^{-1}), the glucose concentration in the fresh medium (between 25 to 50 mM) and its consumption rate, the gradient of dissolved oxygen, the ammonia concentration in the bioreactor (max 25-30 mM), the amounts of added base (1 M NaOH, max 50 mM/day), the lactate concentration in the reactor (max 15-25 mM) and, most crucially, the pH (that must remain higher than 6.1).

6.5. CONCLUSION

In conclusion, we demonstrated the feasibility of the cultivation of High-Five and Sf-9 cells in various packed-bed configuration. The biomass inside the fixed-bed is difficult to assay directly but could be estimated on the basis of volumetric consumption rate of nutrients if a prior calibration has been done. Nonetheless, it is recognized that the lack of direct estimation of the immobilized cell density could remain a problem for industrial applications.

For both cell lines, the cultivation of cells in a rich medium on non-woven fibres in a packed-bed configuration at 0.040 g support/ml of bed and on macroporous carriers (BioNOC II, Fibra-Cel) at 0.067 g support /ml of bed gave good growth and production. A more compact packed-bed configuration increased the critical paths and the heterogeneity of the fixed-bed. During the cultivation, the perfusion rate and the glucose concentration of the medium and the addition of base must be adapted to prevent the excessive accumulation of by-products and dissolved CO_2 and the inappropriate decrease of pH. As for the

infection phase, the perfusion rate must be the lower possible to prevent the dilution of the product titre, while at the same time ensuring the nutrition of infected cells.

Insect cells immobilized on such supports have a specific productivity of r-protein similar to that of suspension culture. Hence, due to the higher cell density obtained in fixed-bed cultivations, their r-protein titre attained was higher than in suspension culture, nevertheless, with a longer time of harvest.

Such fixed-bed cultivations are cheaper, easier to apply and less harmful for cells than classic perfusion (with a cell retention system). Moreover, several supports, like Fibra-Cel and BioNOC II are approved for GMP process and validated by the FDA (American Food and Drug Administration) and the EMAE (European Medicines Agency). Thus, fixed-bed cultivations of insect cells could become an alternative to batch and classic perfusion in industrial applications, including future human therapeutic drugs and vaccines.

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& Conclusion Perspectives

Having arrived at the end of this work, let us recall the context and the goals of our thesis before trying to briefly highlight several experimental results. The major conclusions of the work described in the previous chapters are set out. For each chapter, perspectives, obvious applications and directions for future works are proposed, before ending this work with some general conclusions about the High-Five cell line and perspectives for continuation of research and for large-scale applications of insect cell culture.

AIM OF THE PRESENT WORK

The Sf-9 and High-Five cell lines are the most commonly used for the production of complex recombinant glycoproteins using IC-BEVS. Sf-9 cells are the first cell line used in industrial processes and were considered the most productive cells in the 80s and 90s. Their metabolism is well-known and many media and processes (batch, fed-batch, fixed-bed and perfusion) have been developed specifically for them. However, they generally produce lower levels of r-proteins than High-Five cells that are recognized today as the most productive insect cells. Moreover, High-Five cells have better post-translational modifications than Sf-9 cells (Joshi et al., 2000; Joosten and Shuler, 2003) and have shown superior glycoprotein secretion capacity (Davis et al., 1993). Unfortunately, up to now, relatively few data have been available about suitable environmental conditions and the metabolism of this cell line, a key requirement for the rational development of new processes of mass-production of r-proteins.

Thus, in this context, the overall goal of the present work was the study of the metabolism and the physiology of these High-Five cells as well as the impact of the environmental conditions on their cultivation, in order to develop sophisticated new processes, alternatives to batch cultivation, specifically adapted to this cell line, ensuring high r-protein production titres.

This work was performed in the context of the BEVS for protein expression, using High-Five and Sf-9 cells to compare our new results on High-Five cells to the available literature on Sf-9 cells. Both cell lines were subcultivated in shake-flasks, adapted and cultivated in our own serum- and protein-free YPR medium (Ikonomou et al., 2001) to which dextran was added (Chapter 2). Two recombinant AcMNPV baculoviruses, coding for β-gal and seAP, were employed for infection studies. Bioreactor studies were carried out in a stirred Celligen-Plus™ bioreactor (New Brunswick Scientific, Edison, NJ).

GENERAL CONCLUSIONS AND PERSPECTIVES

INSECT CELL PHYSIOLOGY

Effect of dextran on cell ring formation

At first, we undertook a study aiming at the modification of YPR to prevent cell ring formation on cell culture recipient walls (Chapter 2). This cell ring, mainly composed of dead cells, is deleterious for the cultivation. It contributes to a decrease in the number of free cells and decreases the overall viability. By searching to remove cell clumps in insect cell suspended cultures, we discovered fortuitously that the addition of an optimal type (67 kDa) and concentration (1 g/l) of dextran into the culture medium prevents insect cell ring formation on shake-flask and bioreactor walls. This phenomenon results from the adsorption of dextran simultaneously on the recipients' inner surfaces and on cell membranes, thus inhibiting the complex cumulative and indirect effect of cell adhesion on the vessel walls. Dextran is an inexpensive neutral polymer, very effective in decreasing cell ring and non-inhibitory towards viral infection using High-Five cells and YPR (Ikonomou et al., 2001) or Insect-Xpress (Cambrex, Walkersville, MD) serum-free media. Overall consequences of the decrease of cell ring are the increase of free cell concentration and viability in the medium. When adapted and cultivated in media supplemented with dextran, High-Five cells are bigger and are able to reach higher cell densities (ca. 9-10x10^6 cells/ml) with a high growth rate ($\mu \approx 0.025\ h^{-1}$). With Sf-9 cells, the effect of dextran is less marked due to the fact that these cells are less prone than High-Five to cell ring formation.

Therefore, we recommend the use of dextran-supplemented media for the cultivation of High-Five cells in suspension. We have chosen such media for High-Five process development in Chapters 5 and 6. Moreover, we advise to try dextran addition to medium for suspension cultures of other insect or animal cells that suffer, as High-Five cells, from cell clumping and ring formation.

Insect cell death

The next step in the pursuit of the development of high-density High-Five cell systems was the evaluation of methodologies for cell death assessment and the characterization of environmental conditions affecting insect cell death (Chapter 3). Until now, only a few methods able to detect insect cell death were reported. Thus, we first developed and compared approaches able to distinguish apoptosis from necrosis of insect cells. Among these various methodologies compared, we found that staining of cells with fluorescent dyes (acridine orange, annexin-V-FITC, calcein AM, ethidium homodimer, propidium iodide and rhodamine 123) allowed to easily quantify insect cell death and to classify cell types (viable, apoptotic and necrotic) upon cultivation. Unfortunately, these methods proved to be rather subjective. On the contrary, the use of qualitative methods (ELISA DNA fragmentation, "DNA ladder," caspase detection) was more trustworthy but less accurate and more time consuming. Thus, we advise to use at a minimum more than one method to detect insect cell death, if possible one qualitative and one quantitative. These methods helped us to understand what conditions cause cell death in insect cell cultures but could also be used to better characterize apoptosis pathways.

Among these conditions, a wide range of moderate chemical, physical and metabolic stresses affecting directly the way cells die were tested: for instance, variation of temperature, agitation, concentration of Pluronic F-68, change of pH, availability of nutrients (glucose, Gln, etc.), presence of by-products (lactate, ammonia), etc. Among them moderate chemical and physical stresses (increase of shear stress, modification of pH) triggered a shortening of the growth phase and caused cell death by necrosis, but did not really seem to affect apoptosis, except the variation of temperature. On the contrary, moderate metabolic stresses such as the removal of hydrolysates such as Yeastolate and Primatone, the deprivation of glucose, the presence of the by-product ammonia, all seemed to induce apoptosis. However, lactate accumulation did not cause cell death but seemed to decrease cell growth. We were able to show that the antioxidant activities of Yeastolate and Primatone could explain why their absence caused programmed cell death. High-Five cells were shown to be more affected by nutrient deprivation, ammonia accumulation and thermal shocks than Sf-9 cells, but more resistant to hydrodynamic shocks. In Sf-9 and High-Five cell cultivation, the lack of glucose and the production of ammonia was the main metabolic factors that trigger apoptosis.

Therefore, for the subsequent process development with High-Five cells (Chapters 5 and 6), we showed that we need to confront increased hydrodynamic stress,

glucose-depletion and ammonia accumulation if we want to avoid cell death. Moreover, we showed that we need to sustain the levels of hydrolysates to prevent cell apoptosis.

The investigation of such methods able to distinguish apoptosis and necrosis of Sf-9 and High-Five insect cells opens several perspectives. For future research, such methods could be extended to other Bm, Sf and Tn insect cell lines during the growth and infection phase. Such methods could be used to better understand, what are the critical environmental conditions during insect cell cultivation. They could also be used to develop monitoring of insect cell death by apoptosis and necrosis during cultivation in order to better control cell growth and r-protein or virus particles production. Moreover, such methods could help to investigate the apoptosis pathway in insect cells, which is known to be different than in mammalian cells and today, again, partially unknown.

INSECT CELL METABOLISM

The next step of our work was the study of catabolism and anabolism of insect cell to make up for the lack of knowledge on High-Five cell metabolism (Chapter 4), which is required for process development adapted to this cell line (Chapters 5 and 6). The "extended" metabolism of infected and uninfected High-Five and Sf-9 cells was analyzed by using a metabolic flux network, in order to compare them. The strategy was as follows: firstly, the metabolic pathway network was built on prior published information on insect cell metabolism. Next, experimental kinetics studies were done to obtain data on extracellular consumption and production of metabolites under various environmental culture conditions. These were directly used in the flux network to formulate the catabolic pathways. Furthermore, experimental measure of biomass composition of the cells were used jointly with literature data to determine the anabolic intracellular fluxes.

The analysis of this flux network revealed differences, not previously observed, between the central metabolism of High-Five and Sf-9 cells. High-Five cells have higher metabolic activities than Sf-9 cells. They produce more lactate and ammonia and less Ala than Sf-9 cells. They are able to consume lactate and Ala to provide energy when the other nutrients are deficient. High-Five cells consume preferentially Asn whereas Sf-9 cells prefer Gln/Glu. This Asn is the amino acid the most rapidly consumed by High-Five and its depletion seems to coincide with the onset of the stationary phase (Yang et al., 1996). However, supplementation of Asn in High-Five cell culture does not extend the growth phase, but increases its consumption rate (Chapter 4). Upon infection, High-Five cells use more glucose and less amino acids to produce ATP, compared to the growth phase.

An analysis of the flux metabolic network coupled to an experiment utilizing Asn^{14}C allowed to characterize the metabolic pathway of consumption of Asn by High-Five cells: first Asn is converted in the cytosol to Asp, that enters the mitochondrion and feeds the Krebs cycle after conversion to oxaloacetate.

Our analysis of the metabolic network of Sf-9 cells that shows similar observations as previously published kinetic and metabolism studies, is a good indication of the accuracy of our methodology. If the pathway network is correctly constructed and if we have sufficiently accurate experimental flows and good estimation of biomass content, the use of such a metabolic flux network is a good way to investigate the complete metabolism of cells in vitro under various conditions. This metabolic network made possible a connection between cell catabolism and anabolism and could be used to estimate the energy production by the cells and the amount of nutrients required for r-protein production, for biomass synthesis and for cell maintenance.

In the portion of the thesis dealing primarily with process development (Chapters 5 and 6), we implemented this new knowledge on High-Five cell metabolism to develop better processes: High-Five cells consume high amounts of Asn, Gln, glucose and oxygen; they produce relatively high amounts of ammonia and lactate. If excessive levels of these nutrients are provided to the cells, they waste energy by producing lactate and Ala. In the presence of high lactate concentration, cell growth rate decreases. High-Five cells prefer Asn to Gln but Asn supplementation does not extend the growth phase. Accumulation of lactate is a stress indicator in High-Five cells.

Such a metabolic flux network based on the reconstruction of the cells'metabolic pathways, based on experimental specific consumption/production rates of nutrients and by-products and on the estimation of biomass composition, could be applied to other animal (insect or mammalian) cell lines in suspension culture in order to study their complete metabolism. This methodology could make a possible connection between cell catabolism and anabolism allowing to estimate the amount of energy and of nutrients required by the cells for r-protein production, for cell biomass synthesis and for cell maintenance. Moreover, for future research, such an integrated set of metabolic pathways linked to experimental kinetic data could be used to elaborate a dynamic predictive model that addresses the catabolic and anabolic pathways of insect cells in our case, or, more generally, of animal cells. Such models could help to monitor insect/animal cell cultivation in large-scale industrial processes.

INSECT CELL PROCESSES

The next step of our work was the application of the knowledge on the metabolism and physiology of High-Five cells gained throughout the previous Chapters (2, 3 and 4) to develop fed-batch (Chapter 5) and fixed-bed (Chapter 6) processes specific to High-Five cells, as an alternative to perfusion. We did not take an interest in microcarrier cultivation or in perfusion culture because these aspects have already been studied by several authors (Chico and Jäger, 2000; Ikonomou , 2002; Ikonomou et al., 2002).

Feeding strategies for insect cells

First, we undertook a study aiming at a feeding strategy specific to High-Five cells, which would be a simpler and cheaper alternative to perfusion. The goal of this study was neither the separate increase of cell density nor the boosting of specific cell productivity but the overall improvement of the production titre that incorporates both of them together. Our methodology to develop a fed-batch strategy specific to High-Five cells was based on a factorial experiment to determine what are the nutrients that cells lack at the end of the cultivation, before infection. The use of this statistical method was vital to find that cells need energetic nutrients (glucose, Gln, but not Asn) but also nutrients required for cell biomass (amino acids, lipids, vitamins) and hydrolysates (Primatone and Yeastolate) that prevent a decrease of cell growth. Thus, cells need all media components and we decided to feed them with enriched medium in proportion that are function of their needs. Hence, our next approach was based on an understanding of the cells' requirements during infection and could be summarized in the following questions: what nutrient(s) is(are) needed to be brought in as additive(s) to the cells? when do the cells need it(them)? at what overall level (how much)? how often? and at what individual concentration?

Consequently, we developed an efficient High-Five fed-batch strategy consisting in a pulsed supplement composed of glucose, Gln, Primatone and Yeastolate diluted in basal medium whose formulations evolve as a function of cellular requirements. We found that medium is required during the whole infection phase, whereas hydrolysates are used only at the beginning of the infection, while glucose and Gln are required in decreasing concentration.

This methodology based on a factorial experiment followed by two sets of kinetic experiments, one to determine the cells'needs and to propose a feeding strategy, the other one to optimise this strategy, was very efficient for developing a fed-batch process that increases the cell productivity. Although the methodology was similar for both Sf-9 and High-five cells, the feeding strategy sustaining the highest production titre of r-protein

differs between cell lines. Nevertheless, the strategy sustaining the highest titre, found with High-Five cells, seems not to depend on the r-protein produced (β-gal or seAP). The best fed-batch productions obtained with High-Five cells infected with a baculovirus encoding β-gal or seAP were 5.5 fold higher compared to batch infections. The methodology allowed to obtain higher volumetric productivities compared to a total replacement of the medium by fresh medium upon infection. Similarly, this made better sense than continuous feeding of fresh medium (perfusion) and avoided the use of an expensive and constraining cell retention system.

We have shown that fed-batch processes are particularly interesting for r-protein production using Sf-9 and High-Five cells with the transient IC-BEVS. Although our results were specific to the cell line used (Sf-9 or High-Five) and perhaps specific to the r-protein produced (β-gal or seAP), such methodology based on the understanding of what nutrient(s) is (are) needed by the cells during infection and when the cells need it(them), could be applied to find at what overall level, at what concentration and how often should this(these) nutrient(s) be added, for the development of fed-batch processes using other insect cell lines in suspension culture and producing other r-proteins. Besides, an extension of such a process could be used to investigate the production of viral particles using the IC-BEVS. Moreover, such a methodology could also be applied to develop fed-batch processes with mammalian cells cultivated in suspension that use a transient expression system, which are, just as the IC-BEVS, constituted of two distinct phases (cultivation phase and infection phase).

Fixed-bed cultivations of insect cells

The last step of our work was the application of the previously gained knowledge to the elaboration of a perfused fixed-bed reactor system, that uses a cell retention system, adapted to High-Five cells for r-protein production (Chapter 6), as an alternative to classical perfusion. Our experimental scheme was based on the research of the best environmental conditions: what is the best type of support (non-woven fibres or compact macroporous microcarriers)? which type or model (glass fibres, Dacron® PET, Fibra-Cel® or BioNOC II™)? what is the best compactness? what media? what level of DO? etc. Among the different supports investigated, macroporous microcarriers appeared to be a better choice with a compactness of 0.067 g/ml of bed. A packed-bed with higher compactness increased the preferential paths and the heterogeneity of the bed zone. Although the biomass in the fixed-bed was difficult to assay directly, we have shown that it could be estimated based on the volumetric consumption rate of nutrients upon previous calibration.

We found that the perfusion rate as well as the glucose concentration of the medium and the addition of base must be adapted to prevent the accumulation of by-products and the decrease of pH. During the infection, using a rich medium prevented the dilution of the production due to the high perfusion rate. In addition, we found that the key controlling parameters for a packed-bed bioreactor with High-Five cells are the followings (in increasing order of importance): amino acids consumption rate, DO, pCO2, glucose consumption rate and its concentration in the fresh medium, gradient of oxygen, ammonia and lactate accumulation and pH. Based on the metabolism of cells in the packed-bed, we demonstrated the feasibility of the cultivation of High-Five in a perfused packed-bed system, with the best configuration being based on BioNOC II microcarriers (Cesco Bioengineering, Hsin-Chu, Taiwan). These supports allowed to sustain a density of 400.10^6 cells/g of support and a volumetric production level of β-gal, which represented a level 2.5 fold higher that in batch.

We have shown that perfused packed-bed processes could be used both for growth and infection of Sf-9 and High-Five cells, as an alternative to classic perfusion that uses a cell retention system, in the context of the IC-BEVS. Such fixed-bed cultivations allowed to obtain high production titres of r-protein. For future research, such fixed-bed cultivation could be extended to other animal (insect or mammalian) cell lines using a transient expression system for the production of r-protein or viral particles.

To close this general set of conclusions, we have developed a methodology consisting of the study of the metabolism and the impact of the environmental conditions on the cultivation of insect cell lines (the Sf-9 cell line and more especially the High-Five cell line), in order to develop sophisticated new processes adapted specifically to these cells. To reach this goal, we have developed several tools: a metabolic flux network to study the metabolism of insect cells (Chapter 4), a feeding strategy based on the insect cells' requirements (Chapter 5) and an experimental scheme for the development of fixed-bed processes (Chapter 6). Moreover, we have found that cell ring formation produced by High-Five cells in suspension culture could be reduced by addition of dextran in the culture medium (Chapter 2). We have also investigated methods able to distinguish apoptosis and necrosis in Sf-9 and High-Five cell cultures (Chapter 3). All of these tools and methods could have extensive perspectives and direct applications, presented above, other than the limited Sf-9 and High-Five cell cultivation.

During this thesis, we showed several significant differences in the metabolism, physiology and culture conditions of High-Five cells when we compared them to

mammalian cells, but also to other insect cells, such as Sf-9 cells. Cultivated in an appropriate environment, High-Five cells are more robust and have a higher production rate than other insect cell lines. In the context of the use of IC-BEVS, we showed that if we take care of their specificities, we could develop alternative processes to batch cultivation specifically adapted to them. These processes emphasize the benefits of these cells and are able to sustain high r-protein productivities.

CHARACTERIZATION OF THE HIGH-FIVE CELL LINE

Although the acquisition of new knowledge on High-Five cell line was not the only goal of our thesis, all of the lines of evidence described in the text above have improved our understanding of this cell line. Let us generalize and summarize the characteristics of this cell line, since the beginning of their extended utilization in the middle 90's. The High-Five™ cell line that is increasingly used in industry (Chapter 1), is a clone (BTI-TN-5B1-4) of the embryonic Tn-5 cells (isolated from the "cabbage looper" *Trichoplusia ni*) patented in 1994 by Granados. Originally anchorage-dependent, they are today well adapted to adherence and suspension cultivation in bioreactors. However, they are more prone to clumping than Sf-9 cells. High-Five cells are bigger than other common insect cells (15 µm) with wide cell size distribution and a high biomass content (Chapter 4). When infected, they are bigger than uninfected with higher biomass content (Chapter 4). Their optimal cultivation conditions are as follows: temperature of growth: 27°C, temperature of infection: 25-29°C, optimal pH of 6.2 – 6.3, osmolality of 320-385 mOsm/kg (Chapter 1). In these conditions, High-Five is the insect cell line known to have the higher growth rate (0.030 h^{-1}) and able to reach up to ca. $10x10^6$ cells/ml in batch culture (Chapter 2). Moreover, these cells could tolerate high environmental stresses (like variation of pH, shear stress, ammonia accumulation, lactate accumulation as a by-product, variation of DO and pCO2) (Chapter 3). Yet, they are more sensitive to nutrient deprivation and thermal shock than Sf-9 cells (Chapter 3). Accumulation of lactate does not cause their apoptosis, while ammonia accumulation and serum, hydrolysate (Primatone, Yeastolate), glucose or Gln deprivation triggers their apoptosis (Chapter 3).

Concerning their metabolism, High-Five cells have nutrient uptake rates 1.4 to 2.5 times faster than Sf-9 (Chapter 4). They consume more glucose, Asn (Yang et al., 1996) and oxygen (Chapter 4) and produce more ammonia and lactate (Bédard et al., 1993) than Sf-9. High-Five cells prefer Asn to Gln, but Asn supplementations do not extend cell growth (Chapter 4). They produce less Ala and consume comparatively less Gln/Glu than Sf-9 cells (Chapter 4). In the presence of lactate, High-Five cells cultivated in suspension

may produce clumps and their growth phase may decrease (Ikonomou et al., 2001). This lactate seems to be produced when the culture environment is far from optimal conditions. Its detection in culture is an indicator of cellular stress. But its accumulation seems also to stress the cells even further and creates a positive feed-back loop involving an increase of the cell stress (Drugmand et al., 2005). In the case of glucose limitation, cells are able to consume lactate as energy source (Drugmand et al., 2005). With the exception of this work (Chapters 3 and 4), very few data exist on High-Five metabolism compared to other insect cell lines.

High-Five cells are able to grow in serum-free media richer than those for Sf-9 cells. These media are composed of basal media formulations with addition of glucose, amino acids, hydrolysates (usually Primatone and Yeastolate), lipids, vitamins and surfactant (Schlaeger et al., 1993; Ikonomou et al., 2001). However, relatively few media are specifically developed for the High-Five cell line (only 6 commercial media and 2 media in the public domain) (Chapter 1).

High-Five cells represent the most productive insect cell line as a component of the IC-BEVS. They are able to produce up to 10-20 fold more r-protein than Sf-9 cells (depending on the protein), with the highest production reported yield with insect cells in industrial application (300 mg/ml of human collagenase) (Schmidt, 2004). From the beginning, High-Five cells showed a superior capacity for secreted glycoprotein production (Davis et al., 1993) and better post-translational modifications than Sf cells with a higher percentage of complex glycoforms and even sialylation (Joshi et al., 2000; Joosten et al., 2003).

Concerning their industrialization, High-Five cells seem to emerge as the main insect cell line in the development of new processes for the production of r-proteins. Unfortunately, their utilization in commercial applications requires the payment of royalties, which is not the case with other Sf and Tni cell lines. High-Five cells are well adapted for application in mass-production of r-proteins and amenable to scale-up. They can be cultivated in sophisticated processes such as fed-batch, fixed-bed (Chapters 5 and 6), perfusion (Ikonomou, 2002) and in microcarrier cultivation (Ikonomou et al., 2002). However, until this thesis, only few reports had been published on these processes (with this thesis: 2 perfusions, 3 feeding strategies, 2 fixed-beds and 1 microcarrier cultivation) (Chapter 1).

THE FUTURE OF INSECT CELL CULTURE ENGINEERING

Since the development of the Insect Cell Baculovirus Expression Vector system (IC-BEVS), a huge amount of data has been generated on both insect cells and viruses. These viruses are well characterized and parameters of infection are well-identified. Several cell lines are widely studied, lots of media are available for their growth, and their culture conditions are rather well-known. Insect cells are able to produce high r-protein amounts in a simple batch and the BEVS has important advantages such as high genetic stability, ease of use, good quality of r-protein, etc. For these reasons, the IC-BEVS is commonly used to produce a great variety of r-proteins. Today, its spectrum of applications is limited to the production of r-proteins for biomedical research or to produce diagnostic proteins.

In a near future, insect cells and, among them, specifically High-Five cells are promising for the development of new industrial applications including human therapeutic drugs and vaccines (Chapter 1).

Unfortunately, some unexplored areas in the IC-BEVS remains to be solved, especially concerning the cell density effect and the monitoring of insect cell culture. Moreover, the challenge of therapeutic use of insect cell products imposes more stringent requirements in addition to specific post-translational modifications, such as high levels of biosafety, high productivities as well as high quality standards that have to be maintained all along the production process and from run to run. Let us present here, some initial thoughts.

The consequence of cell density effect on production titre remains not fully understood. As a reminder (Chapter 1), in batch culture, when cell density increases, the specific productivity decreases if the cell density at infection exceeds 2-6.10^6 cells/ml (function of the line used). This phenomena is, in part due to the lack of nutrients. Thus usually a total or a partial change-over of medium is done before or during infection. This change of medium, brings nutrients (glucose, amino acids, etc.) to cells, removes some deleterious by-products (ammonia, lactate, CO_2, etc.) accumulated during the culture, and removes proteases (secreted by the cells, themselves) that can alter the quality of the r-protein produced. Unfortunately, this change of medium, removes also the beneficial autocrine factors, present in conditioned medium (growth promoting factors, anti-apoptotic factors, etc.), that cells have produced during the culture (Calles et al., 2006). Hence, a compromise must be made. Even though, more reports have treated these aspects in the last years, more data must be collected on the compromise to be made between the beneficial

effect of conditioned medium and the requirement to bring in new nutrients and remove by-products and proteases, especially in sophisticated processes (fed-batch, perfusion, etc.).

Moreover, the future industrialization of IC-BEVS will require much knowledge on insect cell metabolism, particularly on the environmental conditions affecting the quality and the quantity of r-protein or viral particles produced, but also on the post-translational modification capacity of cells. So, the establishment of genetically modified insect cell lines, on the basis of well-known insect cell lines (e.g. Bm-5, High-Five, Sf-9, Sf-21, Tn-368, etc.), modified to stably express "human-like" glycosylation in combination with the BEVS is a challenge for new applications (Jarvis, 2003). The establishment of insect cell lines that constitutively express "human-like" post-translational modification is a bigger challenge. Today, baculovirus-infected insect cells have usually lower specific rate of secretion of r-protein than mammalian cells that constitutively express secreted r-protein. Therefore the establishment of genetically modified insect cell lines expressing constitutively some heterologous genes implicated in the secretion of proteins is another future prospect. Finally, the construction of baculoviruses with early promoter joined with the establishment of such insect cell lines, expressing "human-like" post-translational modifications, modified to delay the cell lysis, is a challenge to improve the quantity and quality of secreted r-protein.

Concerning bioengineering, more data must be obtained on the scaling-up of insect cells specifically in sophisticated processes such as perfusion, fed-batch, fixed-bed bioreactor, etc. Large scale cultures must be more studied concerning the effects of gases, shear stress, antifoam, etc. to have the lowest variation possible from one culture to another. Moreover, only few data exists for the monitoring insect cells cultures (de Gooijer et al., 1989; Nielsen et al., 1991; de Gooijer et al., 1992; Power et al., 1992; Hensler and Agathos, 1994; Power et al., 1994; Dalal and Bentley, 1999; Ljunggren et al., 1999 ; Sanderson et al., 1999). A great number of mathematical modelling studies of the baculovirus-insect cell interactions exists, but they do not follow the cell culture. Hence, more research could be made to elaborate predictive models able to monitor insect cell in suspension culture during growth and infection phase. Such models could integrate cell metabolism data (consumption/production rate of nutrients and by-products), infection parameters (MOI, TOI, etc.), environmental conditions affecting the production level and the quality of r-proteins or viral particles, and the cell death (apoptosis and necrosis), in order to follow the cell cultivation under various environmental conditions (media, pH, DO, pCO_2, etc.) in various processes (batch, fed-batch, perfusion, etc.). Of course, elaboration of models that

integrate such requirements is unfeasible, but such recommendations could be used to develop process control specifically adapted to insect cells monitoring.

Today, the development of single-use bioreactors for animal cells cultivated in suspension or fixed-bed cultures, e.g. Wave Bioreactor (Wave Biotech, Somerset, NJ), BelloCell (Cesco Bioengineering, Hsin-Chu, Taiwan), Appliflex (Applikon Biotechnology, Schiedam, The Netherlands) or future prospects development by Artelis (Brussels, Belgium), Sartorius AG (Goettingen, Germany), the Wurm's group (Laboratory of Cellular Biotechnology, Swiss Federal Institute of Technology Lausanne, Switzerland; Jordan et al., 2005) appears to have great interests for industries, especially for the production of small amount of r-protein (up to several grams). Their associations with integration of single–use sensors offers great opportunities for GMP applications (D'Aquino, 2006). Hence, such disposable bioreactors have great hopes for large-scale applications because they allow to reduce investment costs and to improve the safety control of industrial applications (no control of sterility before runs, etc.). Up to now, few reports are published on the use of insect cells in such reactors (Chen et al., 2005; Singh, 1999; Drugmand et al., in press), thus more research must be done in this way.

Concerning biosafety, important research remains to be done on media ingredients. Although, insect cell media are essentially serum-free and protein-free, the main ones of them contain components of animal and yeast origin (Primatone and Yeastolate). On the other hand, therapeutic applications increasingly require animal-free or chemically defined media. Hence, the development of protein-, animal-free insect cell media and processes adapted to them, is a challenge to future development of insect cell cultures.

Therefore, for some applications such as vaccine production, insect cells and more specifically High-Five cells have great potential to compete with mammalian cells. However several improvements must been done. Hence, the research on insect cell culture in not yet finished.

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Appendices

Drugmand, J.-C., Y.-J. Schneider and S. N. Agathos (2005) Environmental effects of lactate on High-Five™ insect cell metabolism *in:* F. Godia and M. Fussenegger (eds.) Animal Cell Technology Meets Genomics (pp 91-94), Dordrecht, The Netherlands, Springer.

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Drugmand, J.-C., Y.-J. Schneider and S. Agathos (in press) Modelling of the Metabolism of Lepidopteran Cell Lines *in:* Animal Cell Technology for Cell Products, The Netherlands, Spinger.

Raw data of the metabolic flux network (Chapter 4)

J.-C. DRUGMAND, Y.-J. SCHNEIDER AND S.N. AGATHOS

ENVIRONMENTAL EFFECTS OF LACTATE ON HIGH-FIVE™ INSECT CELL METABOLISM

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Abstract. In batch suspension culture of High-Five™ insect cells without oxygen limitation, we observed that during the growth phase the presence of exogenous lactate in the medium seems to increase its accumulation by the cells and inhibits cell growth. At the end of culture, after glucose exhaustion from the medium, High-Five™ cells are able to use the produced lactate as a carbon source.

1. INTRODUCTION

The use of insect cells for the production of recombinant (glyco)-proteins by the Insect Cell Baculovirus Expression Vector System (ICC-BEVS) is a well-established technology. Among the industrially important cell lines, High-Five™ insect cells cultivated in serum-free medium have shown great potential for the production and can reach high cell densities.

Contrary to mammalian cell culture, insect cells are known to accumulate little lactate during the growth phase. But under stress conditions, these cells tend to produce more by-products (lactate, ammonium, alanine) (Rhiel et al.1997). These insect cells are generally grown in rich medium. However, in such medium cells waste glucose and have a higher rate of lactate production as a by-product. We report here experimental results of lactate effects on insect cell growth and recombinant protein production.

2. MATERIALS AND METHODS

High-Five™ cells were a gift from the Laboratory of Virology, University of Wageningen, The Netherlands. *Autographa californica* recombinant for β-galactosidase (β-gal) was supplied by Invitrogen (Carlsbad, CA, U.S.A.). The serum-free medium used was YPR formulated and optimised by us previously (Ikonomou et al., 2001). Metabolites were determined using a automatic Bioprofile 100 Analyser (Nova Biomedical, Waltham, MA, U.S.A.). β-gal activities were determined according to Miller et al. (1972). The growth and production phases of the cells were studied in 250 ml shake flasks with 50 ml medium at 28°C and 130 rpm. L-lactate sodium salt, D-lactate sodium salt and alls chemicals were supplied from Sigma (Saint-Louis, MO, U.S.A.)

F.Gòdia and M.Fussenegger (Eds.), Animal Cell technology meets Genomics, 91-94.

3. RESULTS AND DISCUSSION

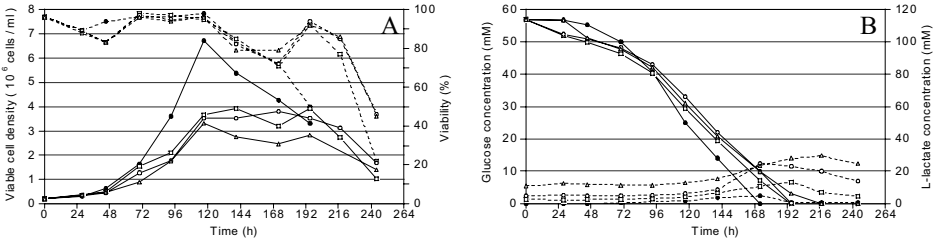

Figure 1: Representative time course profiles of High-Five™ cell density (full lines, panel A), viability (dashed lines, panel B), glucose (full lines, panel B) and lactate concentration (dashed lines, panel B) inoculated in presence of different initial exogenous L-lactate concentration (● control, □ 2.5 mM, ○ 5 mM, Δ 10 mM) in the medium.

We have previously observed that in a fixed-bed culture, immobilized High-Five cells have a different metabolism compared to suspension culture (results not shown). In agitated bioreactor conditions a slight lactate accumulation occurs in the exponential cell growth phase, but in a fixed bed culture, High-Five™ cells tend to produce high lactate concentration apparently due to oxygen limitation. This accumulation of lactate (in lactic acid form) leads to a decrease in the medium pH, thus adversely affecting cell viability. To control lactate accumulation, a strategy of base addition is usually adopted, which increases the pH and buffers the medium but increases its osmolarity.

Nevertheless, in batch suspension culture without apparent oxygen limitation, we observed that the presence of exogenous L-lactate in the medium seemed to increase the lactate production rate and to inhibit cell growth (Figure 1). This inhibition was probably due to the pH decease (uncontrolled) associated with lactate production. At the end of the culture, after glucose exhaustion from the medium, High-Hive cells were able to use the lactate produced as carbon source thus extending the stationary phase. With glucose-limited medium, similar results were observed but with a lower rate of lactate production and lower final cell densities (results not shown). This accumulation and consumption of lactate did not occur with exogenous D-lactate (results not shown).

Table 1: Effects of exogenous L-lactate added in the medium on β-gal production by High-Five™ cells (MOI of 2 at 3.6x10^6 cells/ml) after total change-over of medium before infection.

L-lactate concentration (mM)	Volumetric productivity (Units / ml)	Specific productivity (Units /10^6 cells)
0 (control)	1744	484
5	1401	389
10	980	272
20	543	150

In regard to production, lactate seemed to have different effects than in the growth phase. Without exogenous lactate addition, infected cells produced in the first 2 days post-infection little lactate (3 mM) that decreased pH slightly and then consumed the lactate upon glucose depletion by the end of the infection phase. With exogenous addition, cells directly consumed lactate and no lactate accumulation was observed. With lactate supplementation during infection, cell density and viability decreased more rapidly than the unsupplemented control (Figure 2). This presence of lactate seemed be slightly toxic for the cells and to decrease the recombinant protein production by the BEVS (Table 1).

In this case, the monitoring of lactate concentration and of the pH profile in insect cell culture using BEVS becomes a critical strategy to obtain high production mainly in a process not involving a total change-over of medium before infection.

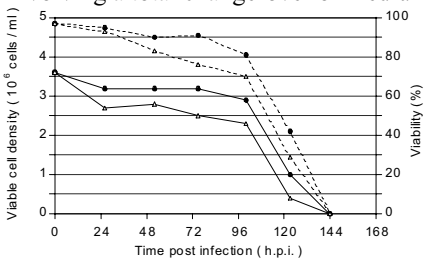

Figure 2: Representative time course of cell density (full lines) and viability (dashed lines) during infection of High-Five™ (MOI of 2 at 3.6x10^6 cells/ml) after total change-over of medium before infection (● control, Δ 10 mM L-lactate).

4. CONCLUSIONS

We can conclude that the presence of lactate in the medium seems to increase the lactate accumulation by growing High-Five™ cells when glucose is in excess. The accumulation may not be due solely to the lack of oxygen, but also reflect a wider repertoire of metabolic responses as a function of pH, lactate and glucose concentration. When glucose depletion occurs, High-Five™ cells are able to use lactate as a carbon source.

The presence of lactate impacts significantly the protein production. Thus, to obtain high productivity, preventing lactate accumulation during the growth phase and before infection is a key point particularly in fed-batch, perfusion and fixed-bed culture.

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GROWTH OF MAMMALIAN AND LEPIDOPTERAN CELLS ON BIONOC® II DISKS, A NOVEL MACROPOROUS MICROCARRIER

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Abstract: The use of BioNOC II microcarriers in a fixed bed bioreactor setup allows to produce high protein levels in a CHO-320 cell line expressing constitutively interferon-γ and also with insect cells used with a baculovirus transient expression vector.

Key words: Fixed bed, Microcarriers, BioNOC, CHO cells, Insect cells

1. INTRODUCTION

Microcarrier technology has the advantages of easy sampling, relatively large surface area and is useful in perfusion culture. Insect cells are well-established for the production of recombinant proteins by the baculovirus expression vector system (BEVS) . CHO cells are widely used for the production of therapeutic proteins. Plant peptones are the safest supplement for protein-free media. They exert a nutritional effect, and they contain bioactive peptides, responsible for increases in growth and production.

BioNOC II™ (Cesco Bioengineering Co, Taichung, Taiwan) cell culture disks are new 100% pure PET nonwoven macroporous carriers for the growth of animal, mammalian and insect cells. BelloCell-500 is a disposable

bioreactor bottle. It consists of a chamber holding 6.5 g of BioNOC II carriers and a lower compressible chamber for mixing and surface aeration by an up/down reciprocal motion.

CHO-320 cells producing recombinant interferon-γ were cultivated in BDM serum-free media (Burteau et al., 2003) supplemented with 0.5-4 g/l cotton peptone from Quest (Naarden, The Netherlands). High Five™ insect cells were cultivated in YPR serum-free media and infected with a AcMNPV baculovirus r-βgal (MOI 2) (Ikonomou et al., 2001). Celligen-Plus 2 L and BelloCell-500 bioreactors were used. The cell biomass was estimated by a protein assay.

2. CHO CELLCULTIVATION IN BelloCell-500

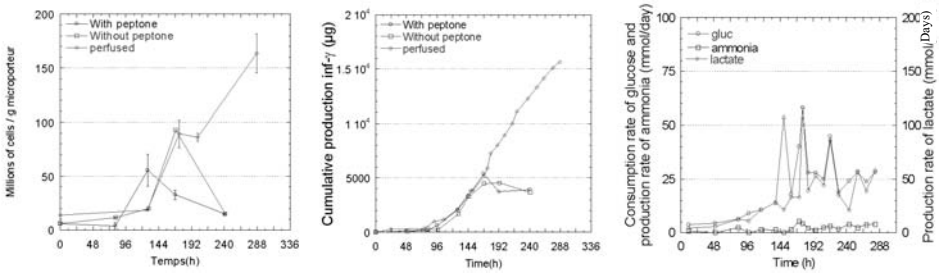

Figure 1. CHO cell cultivation in BelloCell-500. The bioreactor parameters were culture up/down speed = 1/1 mm/s and up/down hold time = 0/3 min.

Two BelloCell-500 batch cultivations were conducted simultaneously with and without peptone (1 g/L). Although the growth was slower (Figure 1), interferon-γ production was slightly higher when peptone was added.

In a BelloCell-500 perfusion cultivation medium was supplemented with 1 g/L cotton peptone. The medium was changed for the first time after 72 hours of culture, when the glucose concentration was low. After 108 hours, medium was changed twice a day. The time course of glucose consumption and of the lactate and ammonia and ammonia production shows that most of the glucose was converted to lactate which decreased the pH, while ammonia production was low. Interferon-γ production was 3-fold higher than in batch.

3. INSECT CELL CULTIVATION IN FIXED BED

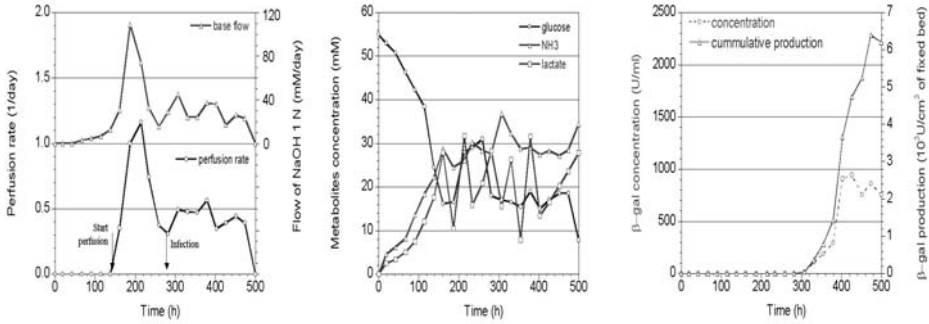

Figure 2. Insect cell perfusion cultivation in Celligen-Plus 2 L bioreactor in fixed bed basket configuration (1.35 L working volume) with 20 g of BioNOC II (bed volume 500 cm^3, 80 rpm, 28°C, 60 % DO). The pH was controlled with the addition of 1 N NaOH.

A fixed bed reactor in perfusion mode was used with BioNOC II. The cells were cultivated for 6 days in batch before starting the perfusion, and the cells were infected after 11 days (Figure 2). The perfusion rate was adapted to keep not only the glucose concentration below 20 mM (a third of the initial concentration) but also to prevent the accumulation of ammonia and lactate above 25 mM. During infection, this perfusion rate was decreased to prevent the dilution of r-protein and keep by-product concentrations in an acceptable range. Lactate production was high (about 1 mol lactate / 1 mol glucose) probably due to the lack of oxygen and accumulation of CO_2 inside the fixed bed. The ratio oxygen / glucose of 3 supports this observation. The pH was controlled to prevent its drop due to lactate production.

Cell density (based on metabolite consumption) was estimated at 400.10^6 cells/g of BionocII before infection.

We obtained after 200 hours post infection (hpi) a high production ($3x10^6$ U β-gal /run) i.e. 2.5 fold higher than in batch. In experiments using the same setup but with Fibra-Cell (New Brunswick Scientific, Edison, NJ), we obtained the same level of production.

4. REFERENCES

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ANALYSIS AND MODELING OF THE METABOLISM OF LEPIDOPTERAN CELL LINES

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Abstract: The network metabolic model of High-Five™ insect cells based on the consumption / production of metabolites and the protein, DNA/RNA, lipid and carbohydrates content of the cells has made it possible to study the internal fluxes of these cells' metabolism.

Key words: Insect Cells, Lepidopteran Cells, Metabolism, Model, High-Five™

1. INTRODUCTION

In the last two decades, the Insect Cell - Baculovirus Expression Vector System (BEVS) has become popular for producing most recombinant (glyco-)proteins. The simplicity of insect cell cultivation in serum-free media and the easy construction of recombinant baculovirus vectors have made the BEVS quite an effective transient expression system. Among insect cell lines, High-Five™ and Sf-9 cells have great potential as technology platforms.

Metabolic flux models are based on the knowledge of the cells' metabolism. In our study, the analysis of pathways was deducted from the literature on insect cells. All concentrations of intracellular intermediates were assumed as constant. We measured the macroscopic extracellular consumption / production rate of each metabolite by enzymatic test or HPLC for amino acids. The cellular content was determined by standard tests

(Bradford test for protein, Hoechst test for DNA, RNA fluorometric test, Soxhlet test for total lipids and the anthrone test for total carbohydrates).

2. ANALYSIS OF THE METABOLISM

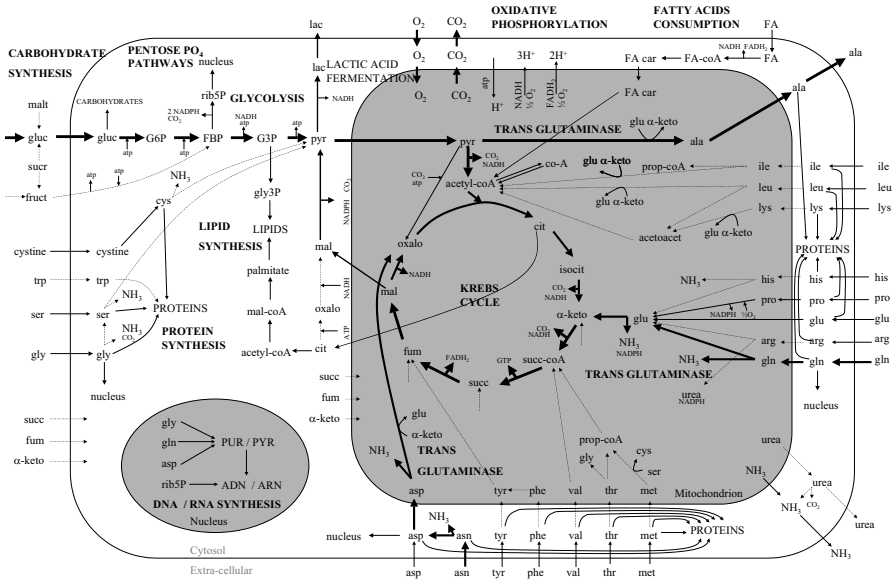

Figure 1. Catabolism and anabolism of High-Five™ insect cells. The thickness of the lines represents the magnitude of the flux

The metabolism of in vitro cultivated insect cells is known to be different from that of mammalian cells. Typically insect cells consume more glucose, oxygen, glutamine (Gln) and asparagines (Asn) than mammalian cells. Moreover, the production of lactate is lower than commonly seen in mammalian cells. The consumption of amino acids and lipids, and the production of ammonia are more intense.

In exponential phase, we found that High-Five cells consume more glucose and Asn than Sf-9 cells. The latter consume more Gln and produce lower lactate than High-Five cells (data not shown).

The consumption of Asn and Gln bring high energy to the cells. Moreover, we found that the feeding of the Krebs cycle by Asn and Gln caused an efflux of malate out of the mitochondrion (15 % of the malate flux), which was converted to pyruvate in the cytosol. In case of cultivation with high Asn (25 mM), this spillover increased by 2.5 times and produced a 3-fold increase of lactate, while keeping the same alanine (Ala) production (Table 1 and Figure 1).

We found that cells consume more nutrients (glucose, Gln, Asn) when we added them in higher concentrations. This may be due to an increase of these compounds' influx when the concentration gradient (between cytosol and media)rises. With high Asn and Gln concentrations, cells consume more glucose, and produce more lactate than in lower glucose concentration. The majority of nitrogen (NH_3 and Ala) produced comes from Asn except in presence of high concentration of Gln. During the lag phase, cells consume high concentrations of amino acid via an anaplerotic pathway (Gln for Sf-9 and Asn for High-Five) and thus obtain high energy levels.In a standard batch culture, we found that the cells used 73 % of the ATP produced for their growth and 27 % for their maintenance.

Table 1. Effect of different media compositions on High-Five™ cells' consumption of glucose, Gln, Asn, the production of lactate and the source of ATP and Nitrogen produced

		High Gluc	Standard Gluc	Low Gluc	High Asn	High Gln	With exogenous Lac
Media composition at the inoculation (mM)	glucose	55	34	12	55	55	55
	lactate	0	0	0	0	0	5
	asparagine	12	12	12	25	12	12
	glutamine	12	12	12	12	25	12
Specific consumption rate of (% of the standard culture)	glucose	**1.15**	**1.00**	**0.88**	**1.37**	**1.45**	**1.39**
	lactate	**0.54**	**1.00**	**1.64**	**1.71**	**3.91**	**1.57**
	asparagine	**1.60**	**1.00**	**0.21**	**3.84**	**1.70**	**1.50**
	glutamine	**0.94**	**1.00**	**0.40**	**2.24**	**15.79**	**2.80**
Ernegy : ATP production (% of the total Atp production)	Krebs Cycle	**66.90**	**60.60**	**59.70**	**66.05**	**56.74**	**71.67**
	Glycolysis	**17.80**	**16.20**	**16.00**	**17.58**	**15.13**	**19.11**
	Lactic fermentation	**0.10**	**0.20**	**0.40**	**2.98**	**0.58**	**0.28**
	Asn consumption	**6.65**	**3.70**	**0.50**	**7.26**	**15.39**	**4.00**
	Gln consumption	**3.80**	**3.40**	**1.40**	**1.86**	**8.73**	**0.80**
	Lipid consumption	3.96	4.10	4.80	3.40	2.99	3.65
	others pathway	0.80	11.80	17.20	0.47	0.45	0.48
Nitrogen production (% of the total Atp production)	Asn consumption	**44.50**	**33.00**	**5.10**	**67.00**	**32.00**	**56.20**
	Gln consumption	**1.90**	**0.50**	**6.50**	**6.20**	**60.00**	**1.80**
	others pathway	53.60	66.50	88.40	26.80	8.00	42.00
Ratio NH3 + urea / ala	ratio NH3 + urea / ala	1.05	1.64	1.93	2.71	2.02	1.23

3. CONCLUSION

The analysis of this metabolic flux network with High-Five and Sf-9 cells has shown different rates in the metabolic pathways in batch cultivation and in infection phase (data not shown) under different operational conditions (e.g. exponential growth phase, lag phase, limitation of glucose, Gln and Asn, etc.).

Based on this flux model, we have constructed a general metabolic pathway network of these insect cells and we have developed a predictive model (data not shown) that can simulate the batch growth, the metabolism (glucose, lactate, Gln, Asn, amino acids, and oxygen) of the cells, plus the time course of the pH and osmolarity during cultivation.

4. REFERENCES

Ikonomou, L., G. Bastin, Y.-J. Schneider, and S.N. Agathos. 2001. Design of an efficient medium for insect cell culture and recombinant protein production. *In Vitro Cell. and Develop. Biol.-Animal,* **37**:549-559.

r = rate, **cyt** = cytosol, **mit** = mitochondrion **out** = extracellular
e.g.: **r X cyt Y cyt** = specific rate of converstion of X present in cytosol into Y present in cytosol

α -keto: α -ketoglutarate, acetoacet: acetoacetate, cit: citrate, FA: fatty acid, FA-car: fatty acid carnitine, FA-CoA: fatty acid-CoA, FBP: fructose-1,6-bisphosphate, fruct: fructose, fum: fumarate, G3P: 3-phosphoglycerate, G6P: glucose-6-phosphate, gluc: glucose, gly3P: glycerol-3-phosphate, isocit: isocitrate, lac: lactate, mal: malate, mal-CoA: malonyl-CoA, malt: matose, oxalo: oxaloacetate, prop-CoA: propionyl-CoA, PUR: purines, PYR: pyrimidines, pyr: pyruvate, rib5P: ribose-5-phosphate, succ: succinate, succ-CoA: succinyl-CoA, sucr: sucrose

specific consumption/production rate of metabolite (µmol/10^9 cells.h)	High-Five growth phase YPR medium	High-Five infection phase YPR medium	Sf-9 growth phase YPR medium	Sf-9 infection phase YPR medium
r ace mit cit mit	231.7	384.6	155.5	165.1
r acecyt malonylcoa cyt	26.9	15.4	23.4	21.6
r acetoace mit ace mit	0.6	0.2	-12.2	1.8
r ala cyt ala out	32.5	46.0	41.4	27.4
r ala cyt protein	10.0	8.5	6.1	2.7
r ala mit ala cyt	32.5	37.5	35.3	24.7
r arg cyt arg mit	1.9	0.5	4.3	1.9
r arg cyt protein	5.7	4.9	3.5	1.5
r arg mit glu mit	1.9	0.5	4.3	1.9
r arg out arg cyt	7.6	5.3	7.8	3.5
r asn cyt asp cyt	17.2	8.8	4.7	2.0
r asn cyt protein	7.2	6.1	4.4	1.9
r asn out asn cyt	30.2	14.9	9.1	3.5
r asp cyt asp mit	20.4	39.7	6.3	4.5
r asp cyt DNA/RNA	1.3	4.7	0.8	0.8
r asp cyt protein	7.2	6.1	4.4	1.9
r asp mit oxalo mit	20.4	39.7	6.3	4.5
r asp out asp cyt	3.3	32.3	5.2	3.6
r cet cyt cet mit	1.0	15.3	10.1	-1.0
r cet mit succoA mit	229.5	434.5	165.3	166.0
r cet out cet cyt	0.5	0.2	0.3	1.0
r cit cyt oxalo cyt	14.2	15.4	23.4	21.6
r cit mit cit cyt	29.9	15.4	23.4	21.6
r cit mit iso mit	202.0	369.2	132.1	143.5
r coa mit ace mit	231.7	384.6	155.5	165.1
r cys cyt protein	2.3	1.9	1.4	0.6
r cys cyt pyr cyt	6.7	2.4	0.2	2.2
r cystine cyt cys cyt	9.2	0.7	1.2	0.5
r cystine out cystine cyt	4.6	0.4	1.0	0.3
r ethanol cyt ethanol	0.2	0.1	0.1	0.0
r FA cyt FAcoa cyt	2.9	2.5	3.5	0.6
r FA out FA cyt	2.9	2.5	3.5	0.6
r FAcar cyt FAcar mit	2.9	2.5	3.5	0.6

	High-Five growth phase YPR medium	High-Five infection phase YPR medium	Sf-9 growth phase YPR medium	Sf-9 infection phase YPR medium
r FAcar mit ace mit	2.9	2.5	3.5	0.6
r FAcoa cyt FAcar cyt	2.9	2.5	3.5	0.6
r FBP cyt G3P cyt	243.4	372.6	195.8	152.3
r fru cyt FBP cyt	2.1	6.1	1.4	3.1
r fruct out fruct cyt	0.0	0.0	0.0	0.0
r fum cyt fum mit	0.1	0.1	0.2	0.1
r fum mit mal mit	228.4	438.8	128.8	177.6
r fum out fum cyt	0.1	0.1	0.2	0.1
r G3P cyt gly3P cyt	1.4	0.8	1.2	1.1
r G3P cyt pyr cyt	244.1	377.9	196.0	154.2
r G6P cyt FBP cyt	129.5	180.2	96.5	73.1
r G6P cyt rib5P cyt	2.4	7.8	1.5	1.8
r gln cyt DNA/ RNA	4.4	15.7	3.1	3.8
r gln cyt gln mit	30.6	46.5	17.0	14.0
r gln cyt protein	4.6	4.4	3.2	1.4
r gln mit glu mit	25.6	40.2	10.6	16.5
r gln mit nh4 mit	8.1	40.2	10.6	16.5
r gln out gln cyt	34.6	66.7	23.3	19.2
r glu cyt glu mit	8.5	1.0	-0.5	6.0
r glu cyt protein	6.3	6.2	4.5	2.0
r glu mit cet mit	27.2	31.9	-15.9	7.6
r glu mit nh4 mit	8.7	31.9	-15.9	7.6
r glu out glu cyt	14.8	13.6	10.4	5.5
r gluc consummed	123.9	164.1	93.5	59.8
r gluc cyt carbohydrate	1.9	0.6	1.5	1.2
r gluc cyt G6P cyt	132.0	188.0	98.1	74.9
r gluc out gluc cyt	133.5	188.6	93.5	76.1
r gly cyt DNA/ RNA	1.1	3.5	0.7	0.9
r gly cyt protein	9.2	7.8	5.6	2.5
r gly cyt ser cyt	5.3	2.5	12.8	-4.6
r gly out gly cyt	2.5	9.5	2.6	2.0
r gly3P cyt lipid cyt	1.4	0.8	1.2	1.1
r his cyt his mit	1.8	0.8	-0.7	0.9
r his cyt protein	3.2	2.7	2.0	0.9
r his mit glu mit	1.8	0.8	-0.7	0.9
r his out his cyt	5.0	3.7	1.3	1.8
r ile cyt ile mit	2.8	0.2	-0.1	0.2
r ile cyt protein	8.1	6.9	5.0	2.2
r ile mit ace mit	2.8	0.2	-0.1	0.2
r ile out ile cyt	10.9	7.0	4.9	2.4
r iso mit cet mit	202.0	369.2	132.1	143.5
r lac cyt lac out	5.8	13.8	4.1	2.7
r leu cyt ace mit	1.0	0.1	0.2	0.0
r leu cyt leu mit	1.0	0.1	0.2	0.0
r leu cyt protein	11.9	10.1	7.3	3.2
r leu out leu cyt	11.9	10.1	7.8	3.2

	High-Five growth phase YPR medium	High-Five infection phase YPR medium	Sf-9 growth phase YPR medium	Sf-9 infection phase YPR medium
r lys cyt lys mit	8.1	0.0	-2.7	2.3
r lys cyt protein	8.7	7.3	5.3	2.3
r lys mit acetoace mit	8.1	0.0	-2.7	2.3
r lys out lys cyt	14.3	7.3	2.6	4.6
r mal cyt pyr cyt	29.8	85.1	18.9	33.4
r mal mit mal cyt	15.3	69.2	-5.2	11.6
r mal mit oxalo mit	211.0	369.6	134.0	166.0
r mal out mal cyt	0.9	0.5	0.7	0.2
r malonylcoa cyt palmitate cyt	26.9	15.4	23.4	21.6
r malt gluc out	7.5	9.2	2.3	6.6
r met cyt met mit	0.2	-1.2	-0.3	2.3
r met cyt protein	2.7	2.3	1.6	0.7
r met mit prop mit	0.2	-1.2	-0.3	2.3
r met out met cyt	2.4	1.1	1.3	3.0
r nh3 cyt DNA/RNA	1.2	3.9	0.8	0.9
r nh4 cyt nh4 out	55.2	38.6	17.9	22.4
r oxalo cyt mal cyt	14.2	15.4	23.4	21.6
r palmitate cyt lipid cyt	26.9	15.4	23.4	21.6
r phe cyt phe mit	0.0	0.1	0.0	2.6
r phe cyt protein	5.7	4.9	3.5	1.5
r phe mit tyr mit	0.0	0.1	0.0	2.6
r phe out phe cyt	5.7	4.9	3.5	4.1
r pro cyt pro mit	1.5	0.8	-0.4	-0.7
r pro cyt protein	6.1	5.2	3.7	1.6
r pro mit glu mit	1.5	0.8	-0.4	-0.7
r pro out pro cyt	7.6	6.2	3.4	0.9
r prop mit succoa mit	0.4	14.0	0.1	10.1
r pyr cyt ala cyt	32.5	37.5	35.3	24.7
r pyr cyt lac cyt	5.8	13.8	4.1	2.7
r pyr cyt pyr mit	261.5	424.4	221.1	179.3
r pyr mit ace mit	231.0	411.7	170.5	160.1
r pyr mit oxalo mit	0.3	4.7	15.3	-5.5
r ribo5P cyt DNA/RNA	2.4	7.8	1.5	1.8
r ser cyt protein	9.5	8.0	5.8	2.5
r ser cyt pyr cyt	2.6	0.4	15.1	-7.8
r ser out ser cyt	6.4	8.0	7.8	1.7
r suc cyt suc mit	0.1	0.0	0.0	0.0
r suc mit fum mit	228.4	443.9	128.7	174.4
r suc out suc cyt	0.1	0.0	0.0	0.0
r succoa mit suc mit	201.0	443.9	128.7	174.4
r sucr gluc out	2.1	6.1	1.4	3.1
r thr cyt protein	7.7	6.6	4.7	2.1
r thr cyt thr mit	0.5	0.4	0.4	-0.4
r thr mit gly mit	0.5	0.2	0.2	-7.9
r thr mit prop mit	1.0	18.7	0.1	7.5
r thr out thr cyt	7.5	6.9	5.2	1.7

	High-Five growth phase YPR medium	High-Five infection phase YPR medium	Sf-9 growth phase YPR medium	Sf-9 infection phase YPR medium
r trp cyt prot	1.9	1.6	1.1	0.5
r trp out trp cyt	1.9	1.6	1.1	0.5
r tyr cyt protein	5.1	4.3	3.1	1.4
r tyr cyt tyr mit	0.5	-0.2	-0.1	3.2
r tyr mit fum mit	0.5	0.2	-0.1	3.2
r tyr out tyr cyt	4.1	1.8	5.2	2.0
r urea cyt nh4 cyt	0.4	5.7	-7.7	4.1
r urea cyt urea out	2.2	2.3	0.4	4.0
r urea mit urea cyt	2.4	-0.5	4.3	1.9
r val cyt protein	8.1	6.9	5.0	2.2
r val cyt val mit	0.1	0.5	-3.7	-1.7
r val mit succoa mit	0.1	0.5	-3.7	-1.7
r val out val cyt	8.2	9.2	1.3	0.5
r Ø production	700.2	1272.9	508.7	499.0
r Ø consumption	654.5	1229.2	459.8	486.0
r electron production	2621.7	4924.6	1840.0	1945.1
r FADH	203.8	446.4	132.3	175.0
r NADH	1107.0	2015.9	787.7	797.6
r NADPH	29.3	128.2	10.0	45.9
r proton gradient	3728.7	6940.5	2627.8	
r total ATP production	4139.4	7780.0	2915.1	3060.0
r ATP Krebs	2508.5	5383.7	1727.6	2243.0
r ATP glycolysis	670.6	1431.5	460.4	596.7
r ATP lac fermentation	8.3	13.2	7.1	3.1
r ATP Asn +Asp	153.2	63.8	124.6	6.1
r ATP Gln +Glu	140.7	515.0	486.5	119.3
r ATP lipids	169.7	61.5	54.6	15.3
r ATP other	488.5	311.2	54.4	76.5
Nitrogen percent Glu +Gln	12.2	59.4	63.5	61.8
Nitrogen percent Asp +Asn	49.2	30.1	20.2	11.9
Nitrogen other pathway	38.6	10.5	16.3	26.3
Ratio NH3 +urea / Ala	1.8	0.9	0.5	0.8

	High-Five growth phase YPR medium 25 mM Asn	High-Five growth phase YPR medium 34 mM glucose	High-Five growth phase YPR medium 12 mM glucose	High-Five growth phase YPR medium 25 mM Gln	High-Five growth phase YPR medium 5 mM Lactate	High-Five growth phase YPR medium 5 mM glucose 10 mM lactate
r ace mit cit mit	373.6	437.2	520.5	120.2	393.0	389.4
r acecyt malonylcoa cyt	34.2	13.8	9.6	37.8	60.0	2.9
r acetoace mit ace mit	15.5	11.3	44.0	-6.8	-62.5	32.3
r ala cyt ala out	63.3	19.7	14.7	115.0	25.8	-62.2
r ala cyt protein	12.7	5.1	3.5	14.1	22.3	1.0
r ala mit ala cyt	50.6	4.6	11.1	101.0	3.5	-63.2
r arg cyt arg mit	1.2	0.7	11.2	-2.0	-9.9	8.9
r arg cyt protein	7.3	2.9	2.0	8.1	12.8	0.6
r arg mit glu mit	1.2	0.7	11.2	-2.0	-9.9	8.9
r arg out arg cyt	8.5	3.6	13.2	6.1	2.8	9.6
r asn cyt asp cyt	68.5	29.8	6.5	30.0	33.3	17.9
r asn cyt protein	9.1	3.7	2.6	10.1	16.0	0.7
r asn out asn cyt	85.0	33.5	9.1	40.0	49.3	18.6
r asp cyt asp mit	58.4	28.0	7.5	37.0	29.2	54.7
r asp cyt DNA/RNA	1.6	0.7	0.5	7.0	2.9	0.1
r asp cyt protein	9.1	3.7	2.6	10.1	16.0	0.7
r asp mit oxalo mit	58.4	28.0	7.5	37.0	29.2	54.7
r asp out asp cyt	9.8	1.2	3.1	19.0	8.9	37.4
r cet cyt cet mit	-5.4	-16.1	-23.0	6.7	10.6	-30.0
r cet mit succoA mit	346.2	486.0	591.1	107.4	368.9	438.7
r cet out cet cyt	1.0	0.8	1.5	0.5	1.2	0.2
r cit cyt oxalo cyt	19.2	13.8	9.6	37.9	60.0	2.9
r cit mit cit cyt	34.2	13.8	9.6	1.0	57.0	2.9
r cit mit iso mit	327.4	423.4	510.9	82.3	333.0	386.5
r coa mit ace mit	363.6	437.3	520.5	120.2	393.0	389.4
r cys cyt protein	2.9	1.2	0.8	3.2	5.0	0.2
r cys cyt pyr cyt	7.1	14.1	30.0	4.7	1.9	12.9
r cystine cyt cys cyt	10.8	16.3	20.9	10.5	8.3	0.7
r cystine out cystine cyt	5.4	8.1	10.5	5.3	4.2	0.3
r ethanol cyt ethanol	0.3	0.0	0.1	0.1	0.1	0.2
r FA cyt FAcoa cyt	3.4	4.8	7.7	3.1	6.2	2.3
r FA out FA cyt	3.4	4.8	7.7	3.1	6.2	2.3
r FAcar cyt FAcar mit	3.4	4.8	7.7	3.1	6.2	2.3
r FAcar mit ace mit	3.4	4.8	7.7	3.1	6.2	2.3
r FAcoa cyt FAcar cyt	3.4	4.8	7.7	3.1	6.2	2.3
r FBP cyt G3P cyt	322.9	364.6	412.6	332.7	527.1	15.2
r fru cyt FBP cyt	1.5	1.6	1.9	1.5	18.8	0.6
r fruct out fruct cyt	0.0	0.0	0.0	0.0	0.0	0.0
r fum cyt fum mit	0.1	0.1	0.3	0.1	0.2	0.1
r fum mit mal mit	342.6	629.7	556.6	-91.0	277.3	492.6
r fum out fum cyt	0.1	0.1	0.3	0.1	0.2	0.1
r G3P cyt gly3P cyt	1.8	0.8	0.5	2.0	3.2	0.2
r G3P cyt pyr cyt	322.6	365.5	413.9	332.2	542.7	15.6
r G6P cyt FBP cyt	160.0	180.6	204.4	164.8	244.7	7.0
r G6P cyt rib5P cyt	3.1	1.3	0.9	3.5	5.4	0.6
r gln cyt DNA/ RNA	3.6	2.6	1.8	1.6	11.1	0.5
r gln cyt gln mit	9.9	46.4	34.6	6.5	18.9	22.1
r gln cyt protein	3.7	2.7	1.8	7.3	11.6	0.6
r gln mit glu mit	9.9	45.2	33.7	8.0	14.5	22.1
r gln mit nh4 mit	9.9	15.8	14.0	8.0	-4.2	40.0
r gln out gln cyt	17.2	48.2	38.7	20.8	35.2	23.2
r glu cyt glu mit	6.7	-3.2	-2.6	7.6	-12.2	38.0
r glu cyt protein	5.3	3.8	2.6	10.3	16.3	0.8
r glu mit cet mit	26.6	26.3	14.8	-56.0	2.2	248.9
r glu mit nh4 mit	47.2	128.0	141.0	-5.6	23.7	249.0
r glu out glu cyt	12.0	2.0	1.1	16.5	5.6	20.9
r gluc consummed	149.1	164.7	185.6	154.0	224.3	6.6
r gluc cyt carbohydrate	2.4	1.0	0.7	2.7	4.3	0.2
r gluc cyt G6P cyt	163.1	181.9	205.3	168.3	250.2	7.3
r gluc out gluc cyt	165.5	182.9	206.0	170.0	254.5	7.9

	High-Five gtowth phase YPR medium 25 mM Asn	High-Five gtowth phase YPR medium 34 mM glucose	High-Five gtowth phase YPR medium 12 mM glucose	High-Five gtowth phase YPR medium 25 mM Gln	High-Five gtowth phase YPR medium 5 mM Lactate	High-Five gtowth phase YPR medium 5 mM glucose 10 mM lactate
r gly cyt DNA/ RNA	1.4	0.6	0.4	1.7	2.5	0.1
r gly cyt protein	11.7	4.7	3.3	12.9	20.4	1.0
r gly cyt ser cyt	0.9	-0.6	-6.5	7.8	7.3	-119.3
r gly out gly cyt	17.6	8.0	6.8	3.1	1.9	7.7
r gly3P cyt lipid cyt	1.8	0.8	0.5	2.0	3.2	0.2
r his cyt his mit	0.5	1.3	9.6	-3.6	-4.9	26.4
r his cyt protein	4.1	1.6	1.1	4.5	7.2	0.3
r his mit glu mit	0.5	1.7	6.6	-3.6	-4.9	26.4
r his out his cyt	4.6	3.3	10.8	0.9	2.2	26.8
r ile cyt ile mit	0.7	11.2	2.8	-2.8	21.9	15.0
r ile cyt protein	10.3	4.2	2.9	11.4	18.0	0.9
r ile mit ace mit	0.7	11.2	2.8	-2.8	21.9	15.0
r ile out ile cyt	11.0	15.4	5.7	8.6	97.0	15.9
r iso mit cet mit	327.4	423.4	510.9	82.3	333.0	386.5
r lac cyt lac out	21.6	19.0	40.3	48.2	29.4	52.7
r leu cyt ace mit	0.0	0.0	7.6	0.0	-22.4	13.1
r leu cyt leu mit	0.0	0.0	7.6	0.0	-22.4	13.1
r leu cyt protein	15.1	6.1	4.2	16.7	26.4	1.3
r leu out leu cyt	15.1	6.0	11.8	16.7	4.0	15.4
r lys cyt lys mit	19.2	7.8	14.5	4.2	-8.9	3.1
r lys cyt protein	11.0	4.4	3.1	3.7	19.3	0.9
r lys mit acetoace mit	19.2	7.8	14.5	4.2	-8.9	3.1
r lys out lys cyt	30.2	12.3	17.6	16.4	10.4	4.0
r mal cyt pyr cyt	56.6	60.3	83.7	-60.5	-9.2	310.0
r mal mit mal cyt	37.4	44.9	71.5	-99.0	7.0	207.0
r mal mit oxalo mit	305.2	447.5	545.9	7.4	348.4	445.4
r mal out mal cyt	1.0	1.5	2.6	1.0	2.0	0.5
r malonylcoa cyt palmitate cyt	34.2	13.8	9.6	37.8	60.0	2.9
r malt gluc out	7.5	8.2	9.3	7.7	5.6	0.2
r met cyt met mit	0.8	-1.0	9.8	-2.7	4.6	12.4
r met cyt protein	3.4	1.4	0.9	8.1	5.9	0.3
r met mit prop mit	0.8	-1.0	9.8	-2.7	-1.4	12.4
r met out met cyt	2.6	0.4	10.8	1.0	13.7	12.7
r nh3 cyt DNA/RNA	1.6	0.6	0.4	3.4	2.7	0.1
r nh4 cyt nh4 out	112.6	81.6	66.0	139.5	110.7	104.2
r oxalo cyt mal cyt	19.2	13.8	9.6	37.9	60.0	2.9
r palmitate cyt lipid cyt	34.2	13.8	9.6	37.8	60.0	2.9
r phe cyt phe mit	0.9	5.8	10.2	-0.7	-6.8	12.1
r phe cyt protein	7.3	2.9	2.0	8.6	12.8	0.6
r phe mit tyr mit	0.9	5.8	10.2	-0.7	-17.0	12.1
r phe out phe cyt	7.4	8.8	12.3	7.3	-6.8	12.7
r pro cyt pro mit	0.5	13.6	19.3	2.2	2.8	0.6
r pro cyt protein	7.8	3.1	2.2	13.3	13.6	0.7
r pro mit glu mit	0.5	13.6	19.3	2.2	2.8	0.6
r pro out pro cyt	8.3	16.7	21.5	10.8	16.4	1.3
r prop mit succoa mit	9.2	6.3	1.1	-1.9	-1.7	28.1
r pyr cyt ala cyt	50.6	4.6	11.1	101.0	3.5	-63.2
r pyr cyt lac cyt	21.6	19.0	40.3	48.2	29.4	52.7
r pyr cyt pyr mit	361.5	367.8	429.5	298.8	455.9	162.7
r pyr mit ace mit	310.7	402.1	451.3	122.5	436.0	336.6
r pyr mit oxalo mit	0.2	-38.0	-32.9	75.0	15.7	-110.7
r ribo5P cyt DNA/RNA	3.1	1.1	0.9	1492.0	5.4	0.3
r ser cyt protein	12.0	4.9	3.4	10.9	21.1	1.0
r ser cyt pyr cyt	0.3	-0.5	-6.2	7.5	8.8	-123.5
r ser out ser cyt	4.9	9.3	16.1	2.1	24.6	9.2
r suc cyt suc mit	0.0	0.1	0.2	0.0	0.1	0.2
r suc mit fum mit	348.4	620.3	600.5	-86.0	294.5	490.8
r suc out suc cyt	0.0	0.1	0.2	0.0	0.1	0.2
r succoa mit suc mit	348.4	620.3	600.5	-86.0	294.5	490.8
r sucr gluc out	1.5	1.6	1.9	1.5	18.8	0.6

	High-Five gtowth phase YPR medium 25 mM Asn	High-Five gtowth phase YPR medium 34 mM glucose	High-Five gtowth phase YPR medium 12 mM glucose	High-Five gtowth phase YPR medium 25 mM Gln	High-Five gtowth phase YPR medium 5 mM lactate	High-Five gtowth phase YPR medium 5 mM glucose 10 mM lactate
r thr cyt protein	9.8	4.0	2.7	7.1	17.2	0.8
r thr cyt thr mit	-0.1	6.6	10.0	-10.2	0.8	9.9
r thr mit gly mit	0.8	6.5	3.6	1.7	-17.0	-24.5
r thr mit prop mit	9.3	6.3	1.1	1.8	4.0	25.4
r thr out thr cyt	1.7	10.6	12.7	0.6	-13.8	9.8
r trp cyt prot	2.4	1.0	0.7	1.8	4.2	0.2
r trp out trp cyt	2.4	1.0	0.7	2.6	4.2	0.2
r tyr cyt protein	6.4	2.6	1.8	11.4	11.3	0.5
r tyr cyt tyr mit	-0.9	9.2	16.7	-5.3	0.7	16.5
r tyr mit fum mit	0.9	9.2	16.7	0.5	39.0	16.5
r tyr out tyr cyt	5.5	6.0	8.3	2.5	5.9	5.0
r urea cyt nh4 cyt	4.2	5.2	-17.0	15.1	26.5	-11.6
r urea cyt urea out	4.5	3.3	2.6	5.6	3.3	3.1
r urea mit urea cyt	2.4	0.7	11.0	-2.0	-9.9	8.9
r val cyt protein	10.3	4.2	2.9	2.6	18.1	0.9
r val cyt val mit	1.0	-2.2	0.0	-9.1	4.3	15.5
r val mit succoa mit	1.0	-2.2	0.0	-9.1	-13.8	15.5
r val out val cyt	9.4	2.0	4.8	2.3	-1.4	16.4
r total ATP production	1051.6	1424.8	1647.3	850.0	1393.8	1231.0
r CO2 production	1000.0	1409.5	1644.2	1359.8	2172.8	1309.2
r electron production	3993.5	5616.8	6546.6	-83.0	5085.7	5235.7
r FADH	351.8	594.0	705.8	509.0	364.7	738.1
r NADH	1644.9	2214.4	2567.5	-109.0	2178.1	1879.6
r NADPH	131.8	266.0	318.4	213.0	18.3	569.5
r O2 consumption	5638.4	7831.2	9114.2	1359.5	7263.8	7114.9
r proton gradient	6287.2	8727.8	10158.6	424.5	8121.8	7974.8
r ATP Krebs	4152.5	5210.5	6796.1	240.8	5820.9	562.2
r ATP glycolysis	1105.4	1396.4	1808.2	64.2	1552.4	149.9
r ATP lac fermentation	187.2	34.9	10.2	2.5	22.8	52.6
r ATP Asn + Asp	456.2	43.6	675.2	65.3	325.0	2408.4
r ATP Gln + Glu	117.0	122.2	386.0	37.1	65.3	949.8
r ATP lipids	213.5	418.9	402.4	12.7	296.3	58.2
r ATP other	29.2	1501.2	81.3	1.9	39.2	3793.6
Nitrogen percent Glu + Gln	20.7	9.1	20.4	20.2	34.5	3.4
Nitrogen percent Asp + Asn	72.2	36.4	7.5	25.6	58.6	3.0
Nitrogen other pathway	7.1	54.5	72.1	54.2	6.9	93.6
Ratio NH3 + urea / Ala	1.7	4.1	4.2	1.2	4.3	-1.7