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Research review paper

Insect cells as factories for biomanufacturing

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ABSTRACT

Insect cells (IC) and particularly lepidopteran cells are an attractive alternative to mammalian cells for biomanufacturing. Insect cell culture, coupled with the lytic expression capacity of baculovirus expression vector systems (BEVS), constitutes a powerful platform, IC-BEVS, for the abundant and versatile formation of heterologous gene products, including proteins, vaccines and vectors for gene therapy. Such products can be manufactured on a large scale thanks to the development of efficient and scaleable production processes involving the integration of a cell growth stage and a stage of cell infection with the recombinant baculovirus vector. Insect cells can produce multimeric proteins functionally equivalent to the natural ones and engineered vectors can be used for efficient expression. Insect cells can be cultivated easily in serum- and protein-free media. A growing number of companies are currently developing an interest in producing therapeutics using IC-BEVS, and many products are today in clinical trials and on the market for veterinary and human applications. This review summarizes current knowledge on insect cell metabolism, culture conditions and applications.

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1. Introduction

The systematic *in vitro* cultivation of insect cells has been possible since the middle of the past century. At that time, the main motivation for establishing continuous insect cell lines was the study of insect physiology and the *in vitro* production of baculoviruses for the biological control of insect pests. In the early 1980s however, insect cell culture moved into the mainstream of biotechnology when it became possible to genetically modify baculoviruses. Their use as vectors enabled the heterologous expression of proteins in a lytic system involving the infection of lepidopteran cell lines (insect cell-baculovirus expression vector system, IC-BEVS). Nowadays, the IC-BEVS has evolved into a major technology platform for the manufacture of viral particles and recombinant proteins with applications ranging from biopesticides to animal and human vaccines and therapeutics and to vectors for gene therapy. This prominent position reflects the advantages of both the cell culture component and the vectors used for gene transfer. The practice of insect cell culture *in vitro* is very well established and the application of recombinant baculovirus vectors for abundant production of proteins or for manufacture of viral particles is robust, safe and scaleable (Agathos, 2010; Ikonomou et al., 2003; Palomares et al., 2006). The IC-BEVS is a highly versatile system because it can express gene products from almost any organism (from bacteria to human tissue) and from any cellular location (intracellular, extracellular, periplasmic). Unlike many industrial mammalian cell culture systems, it is based primarily on engineering the vector and not the host cell line. This shortens drastically the time from gene cloning to protein overproduction (weeks instead of months). The development of a wide variety of transfer vectors makes the isolation of recombinant virus a simple process, while the non-infectiousness of the baculoviruses to vertebrates guarantees the safety of this expression system. Compared to other biomanufacturing platforms, the IC-BEVS offers consistently high product titers, posttranslational modifications only slightly narrower than mammalian cells, and the capacity to express multimeric proteins or even several distinct proteins using the same vector, as the baculoviral genome can accommodate large fragments of heterologous DNA. Insect cells are readily amenable to suspension culture and the continuous improvement of cell culture media and additives (Agathos, 2007; 2010) is contributing to reliable and robust scale-up practices for commercial applications.

Over the last several decades, hundreds of insect cell lines have been isolated from more than 100 insect species encompassing more than 6 orders (Lynn, 2001). 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*, *Mamestra brassicae*, *Spodoptera frugiperda* and *Trichoplusia ni* (Murhammer, 2007; O'Reilly et al., 1992). Among them, Sf-9, Sf-21, Tn-368 and High-Five are the cell lines most widely used in industrial applications. These and related cell lines are highly susceptible to infection by *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV) and other baculoviruses that provide the basis for the construction of vectors in the IC-BEVS.

The first line to be intensively used in research and technological applications was Sf-21, an ovarian cell line from the fall armyworm, *S. frugiperda* established by Vaughn and co-workers in 1977. The Sf-9 cell line was derived from Sf-21 (Smith et al., 1983). Tn-368, was derived from ovarian tissues of the cabbage looper, *T. ni* (Hink, 1970). BTI-TN-5B1-4 (with sometimes different spelling) cells are a clone of the embryonic Tn-5 cell line isolated from *T. ni* eggs (Hink, 1970). Granados patented this cell line in 1994 (Granados, 1994) and it was subsequently commercialized under the name "High-Five™ cells", thanks to its capacity of reaching higher cell densities with higher growth rate and higher production rate than classical Tn-5 cells. 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.

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. 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, thanks to its growth and infection characteristics. The latter are able to better amplify the baculovirus (Wang et al., 1992), while High-Five is the insect cell line that typically produces more r-protein, up to 20 fold more compared to Sf-9 (as a 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 are larger and with higher protein content than Sf-9 cells, and their cell size distribution is wider than Sf-9 cells (Drugmand, 2007). However, the cell size depends on medium osmolarity, shear stress, cell state (viable, apoptotic, etc.) (Palomares et al., 2001). In recent years, the superior characteristics of *expres*SF+(SF+), a proprietary cell line derived from Sf-9 cells, have prompted its use in the manufacture of several biologicals, including the influenza vaccine FluBlok (Cox and Hollister, 2009).

Insect cell lines have polyploid chromosomes, and changes in this polyploidy could occur during cultivation (Doverskog et al., 2000; Léry et al., 1999). These cell lines are able to grow over 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, 1998a), and lower susceptibility to growth enhancement by conditioned medium (Calles et al., 2006a; 2006b).

Finally, the tendency of baculovirus-infected cultured insect cells toward production of defective interfering particles ("passage effect") increases with increased cell passage number (Kool et al., 1991; Pijlman et al., 2001; Wickham et al., 1991).

2. Insect cell metabolism

Knowledge of the metabolic requirements of insect cell lines is primordial for the elaboration of new media and for designing effective feeding strategies in order to ensure superior productivities of r-proteins or baculovirus-based products. Despite the numerous individual studies reviewed below, there is a dearth of comprehensive, systems-oriented investigations of insect cell metabolism and only

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Table 1
Summary of differences 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
Industrialization				
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

recently the first quantitative approaches to this end have appeared regarding the Sf-9 (Bernal et al., 2009; Carinhas et al., 2010; 2011) and High-Five (Drugmand, 2007) cell lines.

2.1. Carbohydrates

Insect cell metabolism has some notable differences from that of mammalian cells in culture (Neermann and Wagner, 1996). Furthermore, 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 2). The latter have a lower rate of production of lactate (Bédard et al., 1993) and metabolic regulation appears to differ between the two cell lines.

Glucose is the most important carbohydrate for insect cells (Fig. 1). It is the preferred energy and carbon source (Drews et al., 1995). Insect cells are known to have a higher specific glucose consumption rate than mammalian cells. 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 other carbohydrates as a carbon source, such as fructose and also maltose, following the hydrolysis of the latter dimeric sugar outside the cell (Bédard et al., 1993; Reuveny et al., 1992), but fructose was found to be consumed only after glucose depletion (Ikonomou et al., 2003). Sucrose, which is added in media to adjust osmolarity, was reported not to be consumed in the growth phase by Sf-9 cells (Bédard et al., 1993; Drews et al., 1995; Reuveny et al., 1992) nor by High-Five cells (Rhiel et al., 1997). It was found to only be consumed by Sf-9 cells during baculovirus infection (Sugiura and Amann, 1996; Wang et al., 1993a), although a low level of sucrose utilization was recently reported during Sf-9 growth and an enhanced rate upon infection at low cell concentration (Bernal et al., 2009). Trehalose, the major carbohydrate in hemolymph, was found to not be consumed if other carbon sources are present (Vaughn, 1973).

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Table 2
Metabolic coefficients (μmol/10^9 cells.h) for High-Five and Sf-9 cells in growth phase cultivated in serum-free media under non-nutrient limiting conditions.

Metabolic rates	Growth phase		Infection phase	
(μmol/10^9 cells.h)	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] Bédard et al. (1993).
[b] Kamen et al. (1996).
[c] Palomares and Ramirez (1996).
[d] Rhiel et al. (1997).
[e] Donaldson and Shuler (1998a, 1998b).
[f] Mendonça et al. (1999).
[g] Benslimane et al. (2005).
[h] Drugmand (2007).

Sf-9 cells have a rather efficient glucose metabolism, with 80% of the pyruvate formed being channeled into the TCA cycle (Bernal et al., 2009; Neermann and Wagner, 1996). Most of the glucose-derived pyruvate is diverted to acetyl-CoA and contributes to cell growth whereas a significant part of it leads to alanine and lipid formation — the latter two being the main reactions reducing the efficiency of glucose oxidation in the TCA cycle (Bernal et al., 2009). Recently, Metabolic Flux Analysis (MFA) approaches have been used to explore the central metabolism of Sf-9 (Bernal et al., 2009; Carinhas et al., 2011) and High-Five cells (Drugmand, 2007).

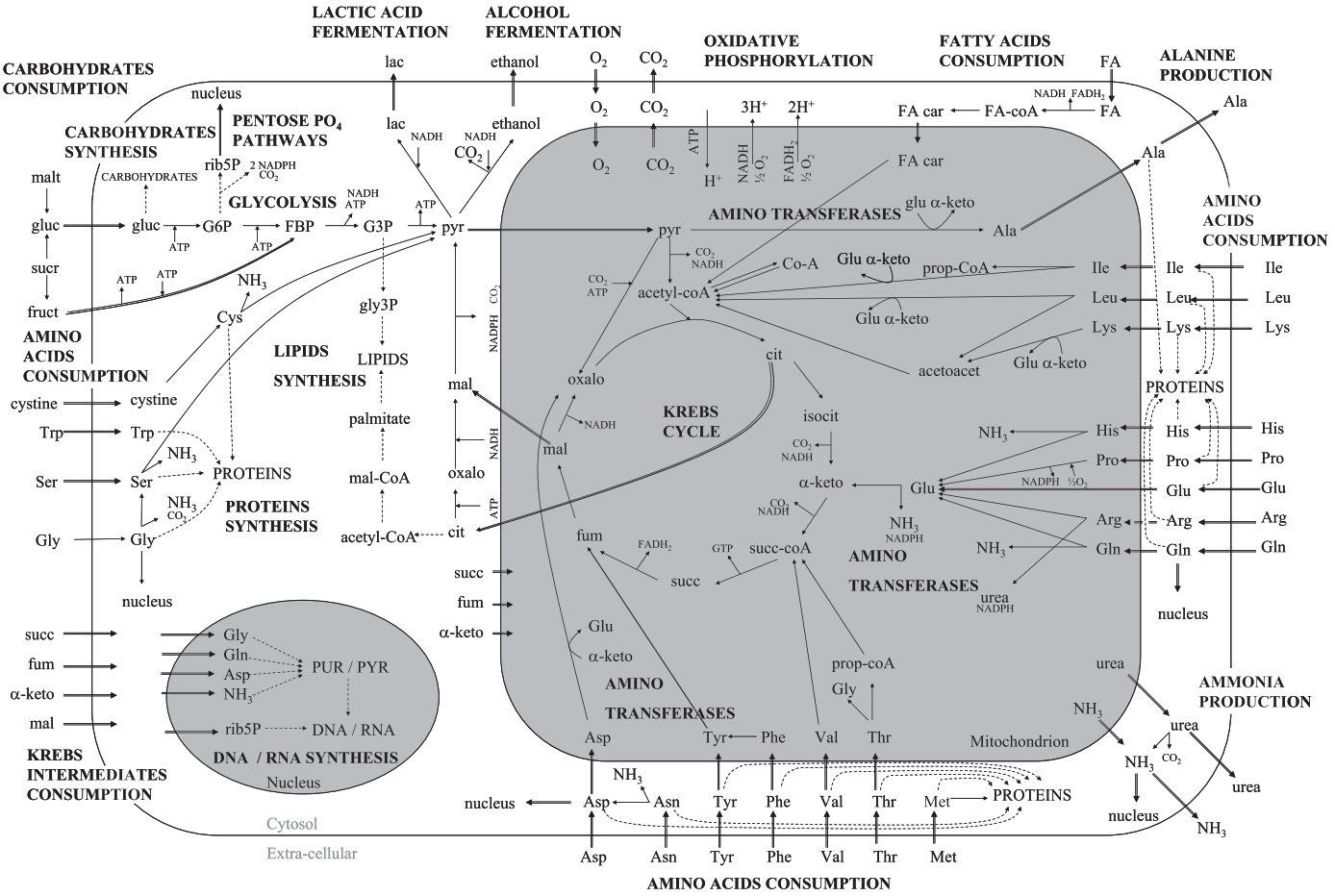

Fig. 1. Metabolic pathways of High-Five cell lines. Full lines, dashed lines and double lines represent, respectively, the catabolic pathways, the anabolic pathways and the transfer of compounds from one cellular compartment to another. α-keto: α-ketoglutarate, acetoacet: acetoacetate, cit: citrate, 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: 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.

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Carbohydrate metabolism in insect cells is regulated similarly to other eukaryotic cells with a fully functional TCA cycle (Bernal et al., 2009; Bhatia et al., 1997). However, specifically in Sf-9 cells, the use of MFA has recently shown (Bernal et al., 2009) that the fluxes through glycolysis and TCA cycle (including anaplerotic fluxes) are downregulated upon increasing cell concentration and that the drop in metabolic energy may be an important determinant of the cell density effect, i.e. the drop in the IC-BEVS productivity when cells are infected at high cell densities (see below).

Under conditions of lack of glucose, the growth of Sf-9 and High-Five cells was observed to stop (Bédard et al., 1993; Drews et al., 2000), but cells can remain viable if enough quantities of amino acids are present. Besides, the absence of glucose triggers apoptosis in Sf-9 cells, whereas High-Five cells can still stay viable by consuming lactate (Drugmand et al., 2005). On the contrary, excessive amounts of glucose result in the accumulation of lactate and alanine (Bédard et al., 1993; Bernal et al., 2009; Mendonça et al., 1999; Öhman et al., 1995). TCA cycle intermediates can be used directly as carbon source but their presence as media ingredients might be useful only in the lag phase. Sf-9 and High-Five cells do not seem to consume them during the growth phase (Drugmand, 2007; Schlaeger, 1996a), and their absence (e.g. in TC-100 medium) has no effect on cell growth (Gardiner and Stockdale, 1975). Recent data confirm that the anaplerotic pathways in Sf-9 cells are less active at high cell densities and after infection, suggesting that under slow growth or non-growth conditions TCA cycle intermediates are not being used for biosynthesis (Bernal et al., 2009).

2.2. Amino acids

Glutamine (Gln) is the main amino acid used as energy source in Sf cultures (Drews et al., 2000; Rhiel et al., 1997). The pathway affecting Gln in Sf-9 cells is highly regulated (Figs. 1 and 2): (Öhman et al., 1996). When glucose is limiting (<7 mM), Gln is converted by glutaminase to glutamate (Glu) thereby producing ammonia. After that, Glu is converted to α-ketoglutarate by glutamate dehydrogenase and feeds into the TCA cycle (Drews et al., 2000; Öhman et al., 1996; Wang et al., 1993a). This pathway is reversible because 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 growth is affected by the absence of Glu suggests that this pathway of Glu synthesis is not completely efficient (Mendonça et al., 1999; Öhman et al., 1996). Under glucose excess, insect cells (especially Sf-9 cells) have a safe way of disposing of nitrogenous by-products (ammonia, see below) in the form of alanine (Öhman et al., 1995).

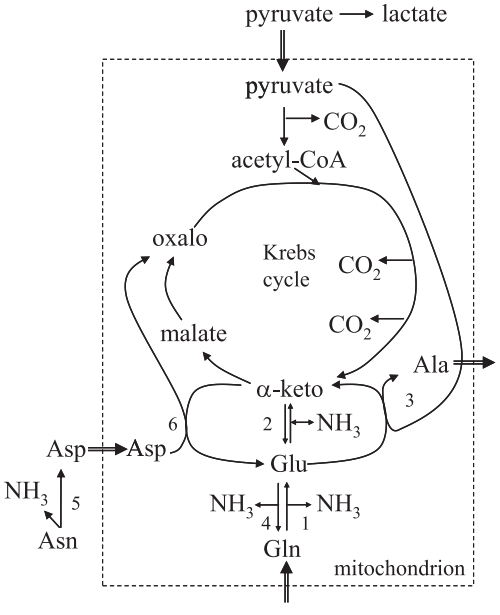

Fig. 2. Gln/Glu and Asn/Asp metabolism in insect cells. Enzymes: 1, glutaminase; 2, glutamate dehydrogenase; 3, alanine aminotransferase; 4, glutamine synthetase; 5, asparaginase; 6, aspartate aminotransferase.

Sf-9 cells display a lower specific rate of consumption of asparagine (Asn) and aspartic acid (Asp) than High-Five (Yang et al., 1996). Asn is consumed extensively by High-Five cells in culture and by Sf-9 cells under conditions of Gln limitation. Asn is used up quickly by High-Five cells (Danner et al., 1995; Rhiel et al., 1997; Yang et al., 1996) 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 seem to extend the growth phase, but instead increases its consumption rate. Asn utilization by High-Five cells is highly regulated and involves enzymes shared between the Gln/Glu and Asn/Asp metabolism (Drugmand, 2007).

The rest of the amino acids are consumed to a lesser extent and their uptake differs from one cell line to another. Arginine (Arg), Asn, Asp, Gln, Glu, methionine (Met) and serine (Ser) are used by Sf-9 for energy production (Drews et al., 1995), whereas Arg, Asp, cysteine (Cys), Glu, histidine (His), leucine (Leu), lysine (Lys), Met, threonine (Thr) can be used as energy source by High-Five cells (Drugmand, 2007).

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 growth. According to 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 the EPa insect line from *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 crucial for Sf-9 cells. Moreover, Cys and glycine (Gly) are essential for Sf-9 cells that do not express methylene tetrahydrofolase (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 (Rhiel et al., 1997; Wang et al., 1993b). 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. It has been recently found that despite the absence of carbon-source limitation in batch cultures of both uninfected and baculovirus-infected Sf-9 cells, amino acid consumption was higher than what was strictly required for protein synthesis; hence anaplerotic pathways were important, especially at low cell densities (Bernal et al., 2009). In this connection, the incorporation of the carbon skeleton of Asp and Asn forms oxaloacetate and that of Gln and Glu forms alpha-ketoglutarate, while the amino moiety is transferred to pyruvate, forming Ala. Ala is an important metabolic by-product (see below) accumulating in insect cells under conditions of glucose excess and consumed upon glucose exhaustion (Bédard et al., 1993; Bernal et al., 2009; Öhman et al., 1995).

2.3. By-products

Insect cell cultures usually accumulate low amounts of lactate in relation to their high glucose consumption, in contrast to mammalian cells (Ikonomou et al., 2003; Neermann and Wagner, 1996). Sf-9 cells do not produce lactate under oxygen-sufficient conditions even in media with high glucose content (>40 mM, e.g., Bernal et al., 2009). In contrast, High-Five cell cultures accumulate higher amounts of lactate, usually between 10 and 20 mM (Sugiura and Amann, 1996; Yang

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et al., 1996). However, if High-Five cells are cultivated in a non-adapted medium (e.g. Sf900-II), accumulation of lactate can reach up to 45 mM (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 to that to mammalian cells due to medium acidification and increase of osmolarity (Cruz et al., 2000). Moreover, the export of lactate out of the cells triggers a decrease of extracellular pH. This toxic effect of lactate up to 10 mM does not seem to affect the growth of Sf-9 cells (Palomares and Ramirez, 1996) while 8 mM of lactate inhibits the growth of Bm-5 cells (Stavroulakis et al., 1991). Lactate accumulation up to 12.5 mM does not trigger necrosis of Sf-9 or High-Five cells, but seems to slightly increase apoptosis (Drugmand, 2007).

In the presence of lactate, High-Five cells cultivated in single-cell suspension may produce clumps (Ikonomou et al., 2001). High-Five cells produce lactate under stress conditions (glucose depletion, shear stress, etc.). Not only 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 type of positive feedback loop (Drugmand et al., 2005). In glucose depleted medium, High-Five cells are able to use accumulated lactate as a carbon source.

Ethanol is another by-product of insect cell glucose metabolism. Its production is usually almost insignificant, but Drews et al. (2000) have reported a production of 6 mM ethanol in serum-free culture of Sf-9 cells. 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 Ala are other important by-products produced from the catabolism of amino acids (Figs. 1 and 2). The toxicity of ammonia on insect cells is not clearly identified, but probably results, as in mammalian cells, from the acidification of the mitochondria and cytoplasm and the modification of the 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 in 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 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 the contrary, High-Five cells were found to produce up to 20 mM ammonia during the growth phase from Gln and Asn metabolism (Chico and Jäger, 2000; Sugiura and Amann, 1996; Yang et al., 1996). Ammonia production depends on the initial Gln and Asn concentrations 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 has been found to affect growth slightly but the productivity of r-protein severely (Yang et al., 1996).

2.4. Lipids

Insect cells have incomplete lipid metabolism and need lipid supplementation. They have a limited capacity of synthesizing, 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).

2.5. Recombinant baculoviruses

In nature, polyhedrin is necessary to form occluded virus (OV), i.e., the only baculoviral phenotype able to survive in the environment and assure larvae infection (Glazer and Nikaido, 1995). *In vitro*, polyhedrin is not necessary for virus survival because budded virus (BV) can readily infect cultured invertebrate cells and replicate in them. Polyhedrin, a protein of 29 kDa, is the major protein in OV that could represent 30–50% of the total larval 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 $1–2\times10^6$ infected cells (Crossen and Gruenwald, 1997; Murhammer, 2007). 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 (Merrington et al., 1997; Smith et al., 1983).

The infection phase of insect cells *in vitro* by a recombinant baculovirus is quite similar to that by wild-type virus. 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 post infection (h.p.i.)), the recombinant virus enters the cell by endocytosis and the nucleocapsid becomes 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 cytoplasmic 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 polyhedrin-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 effects are so extensive that cell lysis occurs, the r-protein is harvested from the culture medium together with virus and cell debris. When the number of virus divided by number of cells (multiplicity of infection: MOI) is less than one, two or more infection cycles are required to infect all cells in the culture.

Genes coding for other viral proteins have also been replaced instead of the polyhedrin gene, for the construction of recombinant baculovirus vectors. 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 to cell lysis (Williams et al., 1989). The use of the *p10* promoter to express a foreign gene helps to delay cell lysis and thus increases 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 capsid protein), etc. allows a more rapid production of r-protein and its harvest from the cells without having to wait 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 has resulted in one of the highest product yields reported, i.e. higher than both with *p10*

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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 process 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 a 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 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 is 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 X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), the r-virus plaques appear colored 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 were introduced. A restriction enzyme site was added on the parental viruses at the insertion locus. These vectors are almost non-infectious. However, when cells are co-transfected with a transfer vector based on the same locus, recombination generates a recircularization 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 in addition to the foreign gene. Today, there are a great 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 amplification steps are usually carried out in Sf-9 cells with a MOI of 0.1 until viral stocks reach 1.10^8 plaque-forming units (pfu)/ml. Nowadays, a huge number of IC-BEVS vector tools are available on the market, some of them for multigene vector generation. These new vectors can be used for the expression of N-terminal and C-terminal protein fusions either cell-associated or secreted into the medium (Yang et al., 2009) (e.g. secreted EPO and human coagulation factor IX) or for production of multiprotein complexes with many subunits (Trowitzsch et al., 2010) (e.g. human transcription factor TFIID, virus-like particles from human papilloma virus serotypes, etc.).

2.6. BEVS as a technology platform in biotechnology

Compared to other systems, the IC-BEVS has several advantages, as summarized by Summers (2006). 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 (Murhammer, 2007; O'Reilly et al., 1992). Yield of production of functional heterologous protein is typically higher than for mammalian cells. Although industrially attained product titers are not often in the public domain, two of the highest reported product titers using IC-BEVS in industrial application were 300 mg/l (human collagenase) with High-Five cells and 80 mg/l (human proapolipoprotein AI) with Sf cells (Schmidt, 2004; van Loo et al., 2001).

Baculovirus vectors allow the expression of large foreign gene products from single proteins under 20 kDa to enzymes and multimeric protein assemblies over one million Daltons (Maiorella et al., 1988; Trowitzsch et al., 2010). Furthermore, this system is adapted for simultaneous expression of several 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 (Trowitzsch et al., 2010). Co-infection with several recombinant viruses is also possible to express different genes (Kost et al., 2005; Rhodes, 1996).

Insect cells are well adapted to serum-free suspension cultivation (Agathos, 2007) and can reach higher cell densities compared to mammalian cells (Kost and Condreay, 1999). They are readily amenable to bioreactor cultivation and scale-up with levels up to 1000 l having been reported (Agathos, 1994; Murhammer, 2007). Insect cells can 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; Hollister et al., 2002; Jarvis, 2003; Joosten and Shuler, 2003; Joshi et al., 2000; Palomares and Ramirez, 2002; 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 reported to carry out better post-translational modifications than Sf-9 cells with a higher percentage of complex glycoforms and even some sialylation (Joosten and Shuler, 2003; Joshi et al., 2000).

Nowadays, the construction of a r-baculovirus is simple, rapid and versatile (Philipps et al., 2005; Trowitzsch et al., 2010; Yang et al., 2009). 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 humans but pathogenic only for insect cells, while the latter do not carry human oncogenes and hence they do not present a cancer risk to humans (Galbraith, 2002; Stacey and Possee, 1996).

The IC-BEVS as a technological concept enables the production of proteins or virus particles *in vitro* in cultured insect cells or *in vivo* in larvae. Although the production yield from larvae is high, industrial r-protein production from larvae is 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.

The IC-BEVS has also some drawbacks. First, it 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 might complicate the purification steps (Murhammer, 2007; O'Reilly et al., 1992). Secondly, post-translational modifications by insect cells are different from those by mammalian cells (Jarvis, 2003; Joosten and Shuler, 2003, Palomares and Ramirez, 2002). Although these generally affect the micro-heterogeneity of the glycoforms, they may alter protein activity.

Due to its transient nature, the duration of protein secretion with the IC-BEVS is shorter than with mammalian cells that express constitutively secreted r-proteins (Schmidt, 2004). Finally, as stated

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previously, mutant viruses tend to appear in viral stocks passaged over many times. These so-called 'defective interfering 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 (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 always 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 provide more complete eukaryotic post-translational modifications, but it generally yields lower production levels. Thus, the IC-BEVS technology constitutes a protein expression platform that is attractive for applications when the benefits are higher than the drawbacks. Further to its well-established usefulness in the production of r-proteins, the IC-BEVS platform is gaining more practical importance for the production of virus-like particles (VLP) as vaccines (Noad and Roy, 2003), baculovirus surface-bound antigens (Xu et al., 2009) and viral vectors for gene therapy (Kost and Condreay, 2002).

3. Insect cell cultivation

Despite the general similarities with mammalian cell cultivation, the cultivation of insect cells has some notable differences (Table 3).

Table 3
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	Moderate (10–20 mM)	High (10–50 mM)
Glucose	Moderate (1–2 mM)	High (2–10 mM)
Amino acids	Often	Yes
Lipids	CO_2/bicarbonate or HEPES or	Phosphate
Buffer	MOPS	Yes (for suspension)
Surfactants	Yes (for suspension)	Not necessary
Serum	Not necessary	More often
Undefined extracts	Less 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 size	Yes	Yes
Cell size upon suspension cultivation	10–12 μm	12–15 μm 14–18 μm when infected

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 (Archambault et al., 1994; Ikonomou et al., 1999; Wu and Goosen, 1996).

3.1. 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 optimum temperature in terms of specific growth rate and final cell density is 27 °C (Reuveny et al., 1993b). 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 thermotolerant. At 25 °C, the specific growth rate (μ) was found to be reduced while at 30 °C, cell viability and maximal cell density decreased (Reuveny et al., 1993a).

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, while their cultivation at 35 °C decreases these parameters (Reuveny et al., 1993a). Increasing the temperature from 22 to 27 °C was also reported to increase the production of β-galactosidase (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). Temperature was also reported to influence the glycosylation potential in the IC-BEVS, as 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).

3.2. Effect of pH and osmolality on insect cells

Concerning pH, values between 6.0 and 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 the CO_2/bicarbonate buffer system commonly used in mammalian cell culture.

The optimum 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), from pH 6.2 to 6.4 for Sf-9 cells (Hensler et al., 1994) and from pH 6.0 to 6.25 for Tn-368 cells (Hink and Strauss, 1976). At lower or higher pH (<6.0 or >6.8), a lengthening of the lag phase and a decrease of the growth rate and maximal cell density were observed. A pH value of 6.2 is generally used for insect cell growth in controlled bioreactors both for the growth and the infection phases. However, the influence of medium pH on r-protein production has not been investigated in detail.

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

3.3. 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 pure oxygen are often used for large-scale mammalian and insect cultures, in order to improve the

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efficiency of oxygen transfer (Marks, 2003). Such small bubbles lead to almost complete dissolution of oxygen which, in turn, is transformed metabolically to CO_2 (1 mole CO_2 produced per mole O_2 consumed, Kamen et al., 1991). However, 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) reported that such a CO_2 accumulation caused a drop in the level of r-protein produced by baculovirus-infected Sf-9 cells. High-Five cells cultivated in a fixed-bed reactor are highly sensitive to CO_2 accumulation and it is recommended to avoid a partial pressure of CO_2 higher than 20% of gas saturation (Drugmand, 2007). In recent work, Bapat and Murhammer (2011) have reported the detrimental effect of high levels of CO_2 on both uninfected and baculovirus-infected insect cells in culture and have suggested the importance of intracellular pH in the inhibitory mechanism.

3.4. Effect of dissolved oxygen on insect cells

Insect cells have a higher specific consumption rate of oxygen than mammalian cells which, as mentioned above, have a higher specific rate of lactate production. Although insect cells need more oxygen than mammalian cells, they can be cultivated in the same bioreactor types with similar gas process control.

Various studies have investigated the effect of dissolved oxygen (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 oxygen consumption rate (q_{o2}) of Sf-9 cells varied with temperature and DO and could be described by a Monod-type equation. During the growth phase, insect cells are not very sensitive to variations in the concentration of DO and can grow optimally in a wide DO range from 30 to 100% (Palomares and Ramirez, 1996; Schmid, 1996).

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, 1994) and Tn-368 cells (Schmid, 1996) are more sensitive to DO than Sf-9 (Agathos, 1996; Hensler and Agathos, 1994) and High-Five cell lines (Donaldson and Shuler, 1998a). Moreover, during this phase, insect cells have a higher oxygen uptake rate (OUR) than uninfected cells (Table 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). Immediately after infection, for both cell lines, a rise of this OUR could be observed (Palomares and Ramirez, 1996; Wong et al., 1994). 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 (Cruz et al., 1998; Taticek and Shuler, 1997). Blanchard and Ferguson (1992) found the highest titer of r-protein was obtained with baculovirus-infected Sf-9 cells at 50% DO. Reuveny et al. (1993a) found that Sf-9 produced more β-gal under conditions where DO was 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. 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. Current attempts to gain more systematic insights into the central energetic metabolism of both uninfected and baculovirus-infected cells through MFA (Bernal et al., 2009; Carinhas et al., 2010; 2011) should lead to a better appreciation of OUR and, therefore, open the way to a more rational control of DO levels for optimized heterologous production.

3.5. Effect of shear stress on insect cells

Insect cells cultivated in bioreactors 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 require high agitation speed in shake-flasks to sustain oxygenation (100–150 rpm) were found to be less sensitive to shear stress than mammalian cells (Chalmers, 1996; Tramper et al., 1986) Insect cell have been found vulnerable to laminar shear above 0.59 N/m^2 and to energy dissipation rates above 2.25×10^4 W/m^3, which is higher than the range of values generated in a classic stirred tank (2 to 3.5×10^3 W/m^3) or bubble column (0.2 to 2×10^3 W/m^3) (Palomares et al., 2006). Cell exposure to high levels of hydrodynamic stress is damaged irreversibly and dies principally through necrosis (Drugmand, 2007). On the contrary, a moderate level of stress implies physiological effects that could trigger cell apoptosis (Chisti, 2000; Tramper et al., 1986).

4. Insect culture media

Insect cell media are different from mammalian cell media. They contain the same basal elements (carbohydrates, amino acids, salt) but with adapted concentrations to insect cell metabolism. For instance, contrary to mammalian cell 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, while antifoam is also commonly added in sparged reactor cultivation (Murhammer and Goochee, 1990). Phenol Red as pH indicator is absent from insect media.

The first insect cell media were developed in the 1960s and 1970s based on the insect cell hemolymph composition. These basal media such as Grace's (1962) medium, 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% fetal bovine serum (FBS). Some media were originally supplemented with hemolymph but serum has become the preferred additive.

Serum provides growth factors, proteins with detoxifying and antioxidant effects, carrier proteins and protease inhibitors and it protects cells from shear stress. Unfortunately, it is expensive, has lot-to-lot variability, supply is limited or unreliable and may contain adventitious agents or contaminants. Moreover, it can interfere with the purification of r-proteins. Insect cells are unable to grow in these basal media (Grace, TNM-FH, TC-100) if serum is removed: they can stay viable, but do not grow. Thus, these considerations,

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among several others, have motivated the development of serum-free media (Agathos, 2007).

Although serum-free medium development can be tedious and costly, substitutes of serum have been sought. Peptones constitute effective 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. 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 1980s. Wilkie et al. (1980) developed CDM that was the first serum-free insect cell medium containing the hydrolysate Yeastolate for growth and infection of Sf cell lines. Yeastolate is a yeast extract ultrafiltered from autolysed yeast biomass that can perform many serum functions. It contains vitamins of the B complex, which are important for the maintenance of insect cell lines (Mitsuhashi, 1989) and it may also contain bioactive peptides (Mendonça et al., 2007). Yeastolate is known to extend the growth phase of insect cells (Bédard et al., 1994; Vaughn and Fan, 1997) and was identified as essential for the production of 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 media IPL-41 and CDM supplemented with Yeastolate (Maiorella et al., 1988), egg yolk (Röder, 1982), lactalbumin (Schlaeger, 1996a) or Ex-Cyte (Hink, 1991) were developed to sustain 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 medium (KDM-10) specifically adapted to Sf-9 cells. SF-1 medium contains Primatone, an enzymatic digest of animal tissue used as a cost-effective supplement which, under serum-free conditions, prolongs the stationary phase by delaying the apoptosis of both Sf-9 and High-Five insect cells (Schlaeger, 1996b). It is to be noted, however, that Primatone is an ingredient of animal origin.

This safety concern has stimulated interest in developing animal-free media. For instance, ISYL is a serum- and animal-free insect medium developed for the large-scale culture of insect cells (Donaldson and Shuler, 1998b). It contains 4 g/l of soy peptone and is based on medium IPL-41. It enables high cell densities and r-protein production with High-Five cells.

A number of commercial media have been specifically developed for Sf and Tn cells and have appeared on the market in the last two decades. Media Sf-900 II, Sf-900 III (with reduced amount of peptones) and Express-Five™ SFM from Invitrogen Life Technologies (Carlsbad, CA), Insect-XPRESS from Lonza (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 used routinely for the cultivation of insect cells. Most current insect cell media are serum-free and contain yeast, meat or soy hydrolysates, the majority being 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. Although culture conditions are not the same for all lepidopteran cell lines, substantial similarities exist (Agathos, 1996; 2007). 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 remain viable in a non-specific medium, they usually need a specific medium well adapted to their physiology and metabolism to sustain high densities and to consistently achieve high production levels. Media developed for High-Five cells are usually richer than those for Sf-9 cells. Often, Sf-9 cells are able to grow in media developed for High-Five cells but with a higher production of by-products (see above). On the contrary, High-Five cells when cultivated in media developed for Sf-9 cells (e.g. Sf900-II) have a reduced growth rate with a high production of by-products and a decrease in gene product yield (Rhiel et al., 1997).

Because the detailed composition of commercial serum-free media is unknown, their use in specific biotechnological applications is not always compatible with the cells' metabolic needs. This is particularly the case in the IC-BEVS which involves a growth phase and an infection/production phase with different metabolic characteristics. A viable alternative is the formulation of low-cost, in-house serum- and protein-free media using empirical, statistical, or genetic algorithm approaches (Agathos, 2007). Fractional factorial design of experiments enables the screening of many ingredients and can achieve time and cost savings as illustrated by Ikonomou et al. (2001) with the formulation of YPR, a serum-free medium based on IPL-41 and supplemented with lipids plus the hydrolysates Primatone and Yeastolate. YPR is efficient in both batch and perfusion cultures with high cell densities and productivities, performing equally well or better than commercial serum-free media that cost 10- to 20-fold more. A next-generation version of this medium that we have found to be as well-performing and cost-effective is YSD (Agathos, 2010). This versatile serum-free medium is essentially YPR to which a low level of dextran has been added and in which Primatone has been replaced by soy protein hydrolysate to ensure that the medium is free of animal-derived components. A genetic algorithm approach in media formulation allows the screening of a great variety of components and each of them at a wide range of concentrations, as shown by Marteijn et al. (2003) who optimized a fed-batch culture of *Helicoverpa zea* insect cells based on 11 different medium components, each used at a range of up to 31 concentrations. The final version of the feed was optimized within four sets of 20 experiments.

5. Bioreactor cultivation

Several bioreactor systems have been studied for robustness and scalability (see reviews by Agathos, 1996; Ikonomou et al., 2003; Palomares et al., 2006). When choosing a production bioreactor, aspects of design (stirred-tank, airlift, packed-bed, or Wave) and of cultivation mode (batch, fed-batch, or continuous perfusion) should be considered in the context of product titer, volumetric productivity, and product quality. Although in the 1990s there was a tendency to experiment with a wide variety of bioreactor designs including unique prototypes, in the last several years there is a tendency to select mainstream reactors, mostly of the stirred-tank type. Published insect cell-based production processes have been extensively reviewed by Ikonomou et al. (2003).

5.1. Batch culture and factors affecting productivity

The IC-BEVS technology involves two distinct phases of cell culture: first the cells are cultivated to reach a desired cell density and subsequently they are infected to produce either r-protein or baculovirus. Infected cells stop growing while uninfected cells continue to proliferate. The culture conditions allowing the attainment of high cell densities in the growth phase are 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 is the principal mode of culture in which cell growth rate is a function of the cell line, the medium and the culture conditions (Schlaeger, 1996a). In this phase, physical and chemical shocks must be kept at a minimum. Batch culture remains the most common method for large-scale IC-BEVS

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processing because of its inherent simplicity and flexibility in bioreactor equipment. However, due to the myriad factors influencing insect cell productivity, departures from batch (esp. fed-batch modes or intermittent medium replacement) before or after infection are routinely practiced and are dealt with further below.

Productivity of infected cells depends on many other qualitative or quantitative parameters 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) and cell energetic state (Carinhas et al., 2010). It is becoming progressively clear that most of these parameters are interrelated.

The fraction of the population of cells initially infected by the virus is a decisive determinant of cell productivity. This fraction is a function of the MOI, which corresponds to the number of infectious particles (plaque-forming units, PFU) divided by the number of cells, and follows a Poisson distribution (O'Reilly et al., 1992). Various reports in the 90s 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 the entire 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). On the other hand, unsynchronized infections increase the time of harvest, the production of defective particles and the exposure of r-protein to proteases (Agathos, 1996). However, different opinions and corresponding practices exist on this point and it appears that MOI cannot be chosen independently from other factors. MOI optimization depends on the virus, the cell line, the media/nutritional conditions and the physiological stage of the cells at infection; hence it is linked to the CDI (Carinhas et al., 2009).

The second main factor affecting production is the cell density effect, i.e. the consistently observed drop in cell-specific productivity when CDI exceeds a certain threshold, e.g., $3–4\times10^6$ Sf-9 cells/ml (Elias et al., 2000) or $5–6\times10^6$ High-Five cells/ml (Ikonomou, 2002). This is a much studied and debated phenomenon that is not yet fully elucidated and has important ramifications ranging from medium and vector selection to bioreactor mode of operation (see below). Usually, this is thought to be a consequence of nutrient depletion in the medium during infection (or, less often, a result of the accumulation of metabolic by-products). Recent insightful work by Bernal et al. (2009) with Sf-9 cells indicates that neither by-product accumulation nor nutrient depletion could fully explain the loss of productivity associated with high cell density infections. The cell density effect appears to have a metabolic basis. Central metabolism is progressively inhibited when Sf-9 cells are grown to high cell densities (caused by incorporation of amino acids into the TCA cycle and the downregulation of glycolysis) (Bernal et al., 2009; Carinhas et al., 2010). Earlier work had already hinted that the drop in productivity of Sf-9 cells occurs prior to nutrient depletion (Doverskog et al., 1997). Nonetheless, complete medium replenishment or selected feeding of nutrients seems to counteract the cell density effect (as reviewed by Ikonomou et al., 2003). Obviously, the selection of the particular strategy to follow in this respect will also depend on the process economics (e.g. media cost).

As stated above, MOI manipulation for improved productivity cannot neglect the role of CDI. For instance, Carinhas et al. (2009) have recently shown that a high MOI delays the fall in production to higher Sf-9 cell densities and that medium exchange at the time of infection using a high MOI has a significant positive effect on baculovirus and r-protein productivity.

Summarizing the experience of many workers in the field, it seems that at high CDI 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. Moreover, the robustness of energetic metabolism in growing and infected cells (Bernal et al., 2009; Carinhas et al., 2009; Carinhas et al., 2010) is emerging as an important consideration for rational improvement of insect cell productivity.

Hence, infections that aim to reach high r-protein or viral particle production must be as synchronized as possible, applied when the cells are highly viable or energetically robust in exponential phase and medium depletion (or ATP generation decline) must be compensated with appropriate nutrient feeding.

Because of the depletion of medium ingredients and the cell density effect, batch culture is not optimal for high protein production levels. The first approach to counteract these drawbacks 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 cell-specific production at high cell density (Ikonomou et al., 2003). Partial medium replacement is less costly and allows the retention of potential growth-promoting factors secreted by the cells (Calles et al., 2006a; Calles et al., 2006b; Doverskog et al., 2000; Ikonomou et al., 2004). Complete medium replacement, originally proposed in the early 90s (Lindsay and Betenbaugh, 1992) can ensure high production with Sf-9 cells but nowadays this solution is neither economical nor practical for large-scale applications.

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 have been applied to Sf cell lines, and fewer to Tn cell lines.

5.2. Continuous culture

Culture in continuous stirred tank reactors (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 IC-BEVS. Thus, continuous operating in a cascade of reactors 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) but its downside is the accumulation of defective virus (van Lier et al., 1996).

On the other hand, perfusion culture, i.e. a continuous culture system equipped with a cell retention device, is more widely used with insect cells because it allows an increase in cell density (Chico and Jäger, 2000). Among the many cell retention systems available, mainly membrane-based devices (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. When cultivated in perfusion, Sf cells have been found to reach very high density, for instance 55×10^6 Sf-21 cells/ml, which is the highest insect cell density reported in perfusion culture (Deutschmann and Jäger, 1994), while the highest density reported with Sf-9 cells was 30×10^6 cells/ml (Cavegn and Bernard, 1992; Cavegn et al., 1992) and 15.5×10^6 cells/ml with High-Five cells (Ikonomou, 2002). The highest published production reported in perfusion mode was ca. 500 mg/ml with High-Five cells (Chico and Jäger, 2000).

Perfusion has the advantage of continuously removing toxic by-products, increasing cell density and reducing the residence time of

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secreted r-protein, hence limiting degradation. It also leads to high volumetric productivity and allows efficient pH and DO control. Unfortunately, high perfusion rates are costly for the 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 stably transformed 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.

5.3. Microcarrier and fixed-bed culture

The use of microcarriers (porous or nonporous) 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 (Griffiths and Looby, 1991, Kong et al., 1999, Looby and Griffiths, 1990, Reuveny, 1990, Rundstadler et al., 1990). There are few reports 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 Tn cells immobilized on collagen-coated microcarriers in a split-flow airlift bioreactor, while 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 the scale-up of adherent cells and increasing considerably their volumetric production, the anchorage-independent nature of most insect cell lines and the one-shot, lytic nature of the IC-BEVS incurs complications 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 microcarrier 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 90s, does not seem to have much of a future for industrial applications due to the heterogeneity of the culture as well as difficulties in harvesting of r-protein and in scale-up (Wu and Goosen, 1996).

Cultivation of insect cells in fixed-bed (packed-bed) reactors consists of an immobilization matrix composed of non-woven fibers, foams or macroporous microcarriers compactly loaded into a retention basket within the reactor (Goosen, 1993). The main advantage of the packed bed is its ability to retain the cells enabling perfusion of the reactor with relatively low shear stress. Moreover, it allows attainment of 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 the culture (Meuwly et al., 2006).

There are only few reports on insect cell cultivation in packed-bed reactors (Chiou et al., 1991; Chiou et al., 1998a; 1998b; Kompier et al., 1991). These packed-bed studies were mainly done with Sf cells immobilized on microdisks or foam matrices. However, High-Five cells seem to also reach high cell densities and high product levels when trapped inside fixed-bed matrices.

In fixed-bed cultivation, the support must satisfy economic 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, immobilization efficiency, growth rate, volumetric and specific production of the immobilized cells and non-adhesion of r-protein to the support) (Archambault et al., 1994; Rundstadler et al., 1990; Wu and Goosen, 1996).

5.4. Fed-batch

Fed-batch operation involves the intermittent addition of nutrients to a batch culture aiming to increase cell density and productivity without medium depletion. It is easier and cheaper to implement than perfusion and avoids exposure of the cells to high shear stress in a cell retention device. 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-product 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 been used to increase production and/or cell density (including, most notably, the remediation of the cell density effect) but some articles have addressed the use of fed-batch to study insect cell metabolism (Jang et al., 2000; Nguyen et al., 1993; Öhman et al., 1995). Bédard et al. (1994) demonstrated, by a factorial experiment, that Sf cells needed a single pulse addition of Yeastolate and amino-acid mixture as supplement to boost productivity. However, the use of a richer supplement consisting of glucose, Tyr, vitamins, iron and trace-metal solution lead to very high densities (30×10^6 cells/ml) (Bédard et al., 1997).

Continuous nutrient addition was found better than single pulse addition leading to the highest reported cell density with lepidopteran cells in fed-batch, at 52×10^6 cells/ml (Elias et al., 2000). However, the cell density effect resulted in unsuccessful infection at such a cell density, while using the same strategy the highest Sf-9 cell density at infection sustaining high production was 17×10^6 cells/ml.

As far as High-Five cells are concerned, few data exist on their fed-batch cultivation. In 1998, Wu et al. attained 4×10^6 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' requirements for glucose and Gln. More recently, we developed a feeding strategy based on an understanding of High-Five metabolism during infection, consisting in pulses of media supplemented with glucose, Gln, Primatone and Yeastolate whose formulations evolved as a function of cellular requirements (Drugmand et al., unpublished). Medium is required during the entire infection phase, whereas hydrolysates are used only at the beginning of the infection, and glucose and Gln are required in decreasing concentrations.

A different approach based on a genetic algorithm was used to optimize the culture media in a fed-batch culture of a *H. zea* cell line (Marteijn et al., 2003). Finally, a recent metabolic dissection of uninfected and baculovirus-infected Sf-9 cells using MFA gave rise to a novel strategy implicating the energetic level of cells for optimal productivity. Medium supplementation with pyruvate or α-ketoglutarate at the time of infection resulted in 6–7-fold higher specific baculovirus yields at high cell density when compared to control cultures (Carinhas et al., 2010).

6. Insect cell technology: applications, market and products

The production of r-proteins and of baculovirus vectors in insect cells is more recent than production of wild-type or modified baculoviruses as insecticides. Lately, the use of baculoviruses as gene

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delivery vehicles has opened exciting new avenues for gene therapy. Today, insect cells in culture are mainly used to produce r-proteins in areas of research and biomedicine and continue to be used for the production of biopesticides. In addition, many products are available on the market for the construction of recombinant baculovirus and for insect cell cultivation.

6.1. Insecticides

The cost of damages inflicted by pest insects and their control is worth US$ 1.25 billion each year in the United Stated alone (FAO: Food and Agriculture Organization of the United Nations). The total world market of baculovirus insecticides accounts for US$ 10 million of sales per year. The first use of baculovirus in agriculture was in 1892 (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 has been estimated that ca. 7 million hectares of crops have been treated with baculoviruses (FAO). These baculoviruses sold as biopesticides could be conditioned in the form of liquids, powders and sprays. Treating 1 ha requires between 10^{12} and 10^{13} viruses (Palomares et al., 2006). The cost of treatment, which was very expensive in the 80s, is today around US$ 0.5/ha (Palomares et al., 2006; FAO). At this cost, viral products are becoming 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 several different pest insect species.

The baculovirus pesticides need to correctly identify the target pest due to their high specificities 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 90s, several recombinant baculoviruses have been 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 sunlight (UV rays) 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 vivo* (in larvae) 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).

6.2. 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). These sectors of application need great amounts of r-proteins. Biological activity and similarity to native proteins represent significant 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; Murhammer, 2007; O'Reilly et al., 1992). Baculoviruses could be also used for production of (secreted) r-protein (e.g. seAP) by transduction of mammalian cells (HEK293 and CHO) (Jardin et al., 2008).

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 (*B. mori*) larvae infected with BmNPV virus but can also use larvae of *T. ni* infected with AcMNPV. This approach is more widespread in Asia where silkworm cultures are well developed (Kost et al., 2005).

Stable insect cell lines (constitutively engineered) are able to produce in continuous mode up to several mg/l of r-protein, sometimes with higher secreted protein yield than the BEVS (Fallon, 1996; Farell et al., 1998; Jarvis, 1991). Despite these advantages, the establishment of stable insect cell lines expressing r-proteins in a non-lytic system does not seem to progress toward industrial applications, because these insect cells generally produce less protein, the time for establishment of a stable cell line is longer and it is more expensive than IC-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 considered more robust, there is more known about their bioprocess behavior, and they possess the capacity for post-translational modifications nearest to human proteins (McCaroll and King, 1997).

6.3. Therapeutics

The efficacy, safety and cost aspects appear to indicate that for some biomedical applications the production of recombinant proteins or viral medicinal agents using IC-BEVS will be useful. The IC-BEVS constitutes a good system for the production of vaccine subunits by expressing antigen proteins or by production of VLP as vaccines (Schmidt, 2004; Schmidt and Hoffman, 2003). A VLP is a virus identical to the native virus expressing surface proteins from one or more

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different pathogenic 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 somewhat different glycosylation 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, The Netherlands). Today, other commercialized veterinary drugs are made using IC-BEVS (Walsh, 2005): Bayovac vaccine from Bayer (Germany), Advasure vaccine from Pfizer and an interferon from Virbac (France).

In the area of human biopharmaceuticals, the fear of new technology and the cost of drug development have been important constraints slowing down their development using insect cells. Studies have suggested that the IC-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 (USA) is planning to use *in vivo* caterpillar larvae infected with a recombinant baculovirus producing H5 hemagglutinin as a veterinary vaccine against the bird flu. They have developed an avian flu vaccine (Influenza H5) *in vivo* using caterpillars.

Cervarix® from GlaxoSmithKline (Belgium) was the first IC-BEVS based human commercial product on the market: a human papilloma virus vaccine. Many therapeutic vaccines and biopharmaceuticals using IC-BEVS technology are currently in the pipeline. Among human vaccines, insect cell-derived influenza vaccines represent valuable tools for the fight against seasonal and especially pandemic flu outbursts (Cox and Hollister, 2009). Protein Sciences has begun the phase III clinical trials of an Influenza type A vaccine, against the most common type of virus causing human flu. They have also a vaccine program against HIV-AIDS based on a similar approach. Flu vaccines based on insect cell-produced recombinant hemagglutinin (rHA) have completed phase III clinical trials. FluBlok®, the first rHA influenza vaccine (Cox and Hollister, 2009), is expected on the market in the course of 2011 (Protein Science; Krammer and Grabherr, 2010). The IC-BEVS is used to generate Novavax, a non-infectious recombinant VLP vaccine from both influenza A H5N1 clade 1 and clade 2 isolates with pandemic potential (Bright et al., 2008). This candidate is in phase II clinical trial (Krammer and Grabherr, 2010). Concerning cancer vaccines, Dendreon Corporation has a prostate cancer vaccine candidate in phase III clinical trials. MedImmune Inc has been working on a human papilloma virus vaccine. GlaxoSmithKline has a hepatitis E vaccine candidate under development using insect cells. Wyeth (now Pfizer) has development programs including production from insect cells of a HIV vaccine, a malaria vaccine and a needle-free flu vaccine. As far as therapeutic drugs are concerned, Protein Sciences will soon bring to human testing erythropoietin (EPO) produced with IC-BEVS. Amgen (USA) and Sanofi-Pasteur (France) have programs of vaccine and therapeutic drug development using insect cells that are in preclinical phase. Human Genome Sciences has under development some therapeutics using insect cells that are in the preclinical and clinical phases. Bayer (Germany), Intervet International, Pfizer and Vibrac are reportedly developing veterinary products using IC-BEVS. Pfizer and Bayer are not disclosing any information regarding the development of human therapeutics based on the IC-BEVS platform. However, their choices to produce veterinary vaccines using this technology indicate an interest in the IC-BEVS platform and perhaps future development of human therapeutics. Other vaccine projects using IC-BEVS are under development by several companies and institutes: veterinary vaccines in preclinical phase against foot-and-mouth-disease and canine leishmaniasis; the development of a human vaccine candidates against HIV-AIDS, malaria, hepatitis A, B, C and E, human hookworm disease, human parvovirus and human polyomavirus (WHO: World Health Organization). Various other biotherapeutics have been or are currently under investigation, e.g. antistasin, erythropoietin, interleukins, tPA, glucocerebrosidase, etc. (Palomares and Ramirez, 1998).

In recent decades, there have been several studies on insect cells expressing "human-like" glycosylation patterns by eliminating "insect like" glycosylation and replacing the insect genes with genes encoding enzymes that mimic mammalian/human glycoform synthesis (Jarvis, 2003, Tomiya et al., 2003). Insect cells stably transformed with mammalian glycosyl transferases (Hollister and Jarvis, 2001; Hollister et al., 1998; Hollister et al., 2002) have given rise to the 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 Life Technologies (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 an 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).

In the area of biosafety, important research remains to be done on media ingredients. As already mentioned above, although insect cell media are nowadays essentially serum-free and protein-free, the main commercial formulations contain components of animal and yeast origin (Primatone and Yeastolate). On the other hand, therapeutic applications increasingly require animal-free and chemically-defined media. Hence, the development of protein and animal-free insect cell media and processes adapted to them, remains an important target for future development of insect cell cultures (Agathos, 2007, 2010).

6.4. Other applications of baculovirus

The middle of the 1990s saw the establishment 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 these macromolecules to the surface glycoprotein gp64 of the AcMNPV. 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, baculovirus display seems to generate less abundant libraries compared to prokaryotic virus display technologies such as phage display (Grabherr and Ernst, 2001; Grabherr et al., 1997).

Baculoviruses started being studied in the 90s 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). The manufacture of modified baculovirus vectors able to direct gene expression in mammalian cells represents a safer alternative over classical mammalian viruses (Bernal et al., 2009). 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 Possee, 1996). Baculoviruses are known to be

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inactivated by the immune system, but direct injection of virus in tissues has allowed delivery of genes into the brain or muscle of mammals (Pieroni et al., 2001; Sarkis et al., 2000). The validation of this approach implies generating knowledge on the mechanisms governing tissue targeting, gene delivery, and host immunological responses to the engineered BVs. Recently, Carinhas et al. (2009) have explored culture conditions (e.g. the role of cell density, multiplicity of infection and medium exchange) required for the production of baculovirus as gene therapy vectors. In order to facilitate the use of BVs in gene therapy, efficient production processes to amplify the engineered vectors will be increasingly based on rational approaches including insect metabolome analysis and metabolic engineering (Benslimane et al., 2005; Bernal et al., 2009; Carinhas et al., 2009; Carinhas et al. 2010; Carinhas et al., 2011).

7. Concluding remarks

In addition to its established applications in the production of 'green' biopesticides and of r-proteins in the research and diagnostics sector, the IC-BEVS platform is enjoying increasing appeal thanks to new applications in human and animal health, including biopharmaceuticals and new-generation vaccines. The technology is also naturally adapted for producing viral vectors for cell therapy and for readily expressing multimeric or otherwise unwieldy proteins of interest in fundamental biological research or in drug discovery, sustainable agriculture practice, etc. The in-depth understanding of both uninfected and infected insect cell physiology using metabolic engineering and the constant improvement of insect cell media and culture techniques using the tools and concepts reviewed should contribute to the rational validation of efficient, scaleable, and safe production processes based on the IC-BEVS technology.

8. Regulatory agencies

American Food and Drug Administration: FDA. www.fda.gov/.

European Medicines Agency: EMEA. http://www.emea.eu.int/.

Food and Agriculture Organization of the United Nation: FAO. http://www.fao.org/.

Public Health Agency of Canada. http://www.phac-aspc.gc.ca.

World Health Organisation: WHO. http://www.who.int.

The Japanese Ministry of Health, Labour and Welfare: MHCW. http://www.mhlw.go.jp/english/.

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