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Production of Insecticidal Baculoviruses in Insect Cell Cultures: Potential and Limitations

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1. Introduction

The potential of baculoviruses to be employed as insecticides is known since more than 75 years ago (Benz, 1986). To date, over 30 different baculoviruses are used to control several insect plagues in agriculture, horticulture and forestry (Moscardi, 1999). The use of baculovirus as insecticides is based on a set of useful properties, such as pathogenicity, specificity, narrow host range, environmental persistence, ability to act synergistically with other natural enemies of the pest and ability to induce artificial epizootics. Despite these advantages, very few baculoviruses have become widely used as insecticides, standing out as some successful examples the use of the *Anticarsia gemmatalis multiple nucleopolyhedrovirus* (AgMNPV) to control the velvetbean caterpillar in soybean crops in Latin America, *Cidita pommonella granulovirus* (CpGV) to fight the codling moth attacks in fruit orchards, and *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV) to control the armyworm in vegetable crops under cover in Europe (Moscardi, 1999). The causes of the limited acceptance of baculoviruses as insecticides are diverse, including slow speed of action, problems to register and market these biological insecticides and difficulties to produce them at an appropriate scale.

The technologies currently used to produce insecticidal baculoviruses are based on the infection of susceptible insect larvae (Black et al., 1997). However, the implementation of processes to produce baculovirus in insect larvae is hampered by several limitations: high labour requirements, lack of expertise in standardization and validation of such processes, difficulties in scaling production to levels consistent with the profitability of the process and difficulties to properly control both the process production and product quality. While several improvements in production systems in insect larvae have been described in the last years which could help overcome some of the problems described above (van Beek & Davis, 2007), it has been also proposed that the adoption of an alternative technology based on the viral propagation in insect cell cultures could enable the development of well standardized, controlled and scalable production processes for insecticidal baculoviruses (Szewczyk et al., 2006).

The purpose of this chapter is to review the current state of the art about insect cell culture technology and its application to the production of viral insecticides belonging to the family *Baculoviridae*. The several restrictions still existing to develop feasible processes as well as the prospects for overcoming these limitations will be also reviewed.

2. The baculoviruses

2.1 Structure and classification

The baculoviruses (family *Baculoviridae*) are a group of arthropod-specific viral pathogens that have a circular and supercoiled double-stranded DNA genome (Rohrmann, 2011). The size of the genome ranges from 80 to 180 kbp. The genome is contained in a rod-shaped nucleocapsid with helical symmetry. The baculoviruses generate two different progenies, called budded virus (BVs) and occluded virus (OVs) that share the same nucleocapsid. Both viral progenies play different roles in the natural cycle of these viruses. BVs, consisting of a single nucleocapsid surrounded by an envelope derived from the cellular plasmatic membrane, are responsible for the transmission of the infection from cell to cell in an infected animal. In OVs, on the other hand, one or more nucleocapsids are contained by an envelope synthesized *de novo* in the nucleus of infected cells. These virions are then included in a crystalline protein matrix, consisting mainly of a single polypeptide which is product of the hyperexpression of a very late viral gene, resulting in the so-called occlusion bodies (OBs). The OBs, whose polypeptide structure gives protection to the OVs contained therein, are responsible for the transmission of the infection between susceptible animals in nature and, in fact, constitute the viral progeny useful as insecticide. The structures of BVs, OVs and OBs are shown in Figure 1.

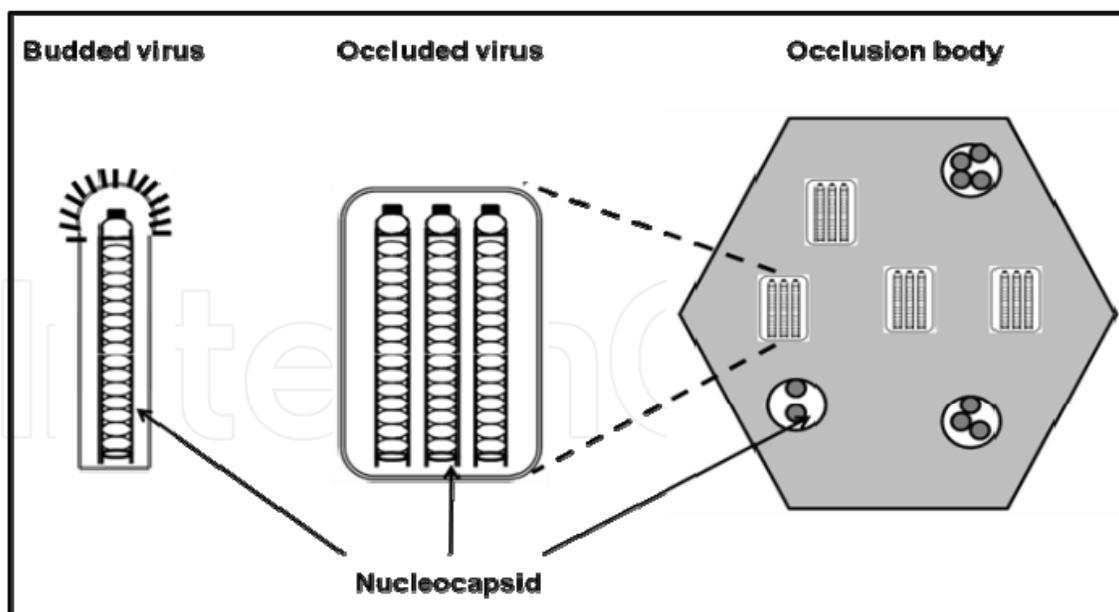


Fig. 1. schematic structures of budded virus, occluded virus and occlusion bodies of baculoviruses.

The members of the *Baculoviridae* family are classified into four different genera (Jehle et al., 2006). The viruses classified into the genus *α-baculovirus* are able to infect lepidopteran insects to produce nuclear polyhedrosis. The viruses belonging to the genus *β-baculovirus*

can also infect lepidopteran insects, but they produce granulosis. On the other hand, the viruses classified into the genus γ -*baculovirus* can infect hymenopteran insects to produce nuclear polyhedrosis, while the viruses of the genus δ -*baculovirus* are associated to the production of nuclear polyhedrosis in dipteran insects. As most baculoviruses used as insecticides so far belong to the genus α -*baculovirus*, the subject henceforth will be focused on lepidopteran nucleopolyhedroviruses.

2.2 Natural cycle and pathogenicity

The main route of infection of lepidopteran larvae with nucleopolyhedrovirus is the ingestion of food contaminated with viral OBs (Granados & Williams, 1986). Once ingested, OBs are transported to the larvae's midgut, where they are dissolved to release the occluded virions, due to the combined action of the alkaline environment and the presence of alkaline proteases. The released OV's pass through the midgut peritrophic matrix and find the brush border membrane of the columnar midgut epithelial cells, which fuse with the viral envelope to enter the viral nucleocapsids within the cytoplasm. The ability of OV's to infect midgut epithelial cells is dependent on the expression of a set of genes whose products are denominated "*per os* infectivity factors" (PIFs) (Rohrmann, 2011). Most nucleocapsids are then transported to the nucleus through a process that is dependent on actin polymerization. Once the nucleocapsid has been entered into the cell nucleus, the viral DNA is naked and starts the transcriptional cascade that lead ultimately to the assembly of progeny nucleocapsids. A distinctive feature of the primary replication of nucleopolyhedroviruses in the midgut is that the nucleocapsids that were assembled in the nucleus are almost totally exported to the basal cytoplasmic membrane, from where they finally egress as BVs. The budding of BVs occurs at regions of the plasmatic membrane that have been modified by insertion of the glycoproteins characteristic of the BV progeny, GP64 and/or F. Then, BVs would cross the basal lamina to begin the dissemination process that leads to secondary systemic infections. Alternatively, it has been proposed that baculoviruses can reach the main insect cavity through previous infection of tracheal cells (Pasarelli, 2011). Secondary infections, that affect almost all insect tissues, start when BVs penetrate into cells through a receptor-mediated endocytosis process. After the fusion of the viral envelope with the membrane of acidified endosomes, viral nucleocapsids are released into the cytoplasm and then transported to the nucleus, where the viral genome is naked. Differently from what occur in midgut epithelial cells, the transcriptional cascade in secondary infections drives the replication process to the production of nucleocapsids that, besides of feeding the budding of BVs, are assembled into the nucleus to form OV's. The assembly of OV's implies the retention of nucleocapsids inside the nucleus and the acquisition of an envelope synthesized *de novo* at the expense of the inner nuclear membrane. The OV's are finally occluded inside a crystalline matrix consisting mainly of the viral protein polyhedrin, whose gene is expressed at very high levels during the very late stage of the transcriptional cascade. The products of the occlusion process are the OBs, which are retained inside the nucleus until the death and lysis of the infected cell. At the end of the pathogenic process, the infected insect is full of OBs that, after its death and liquefaction of its tissues, are released into the environment to restart the cycle again.

The symptoms of the disease associated to baculovirus infection are not usually apparent during the first days post-infection (Granados & Williams, 1986). The change of color and the altered behavior of infected insects are often the earliest signs of infection with α -baculovirus. The lack of appetite, which after several days culminates in the total

interruption of feeding, is another sign of infection. The growth of infected larvae is delayed with respect to uninfected controls, and the death occurs after several days post-infection. The length of the interval of time between infection and death of the insect varies between 3 days and 3 weeks, and depends on many factors, including larval age, nutritional status, dose of virus and virulence of the viral strain, as well as on environmental factors. Anyway, nor the cessation of ingestion or death occur immediately to infection, facts that constitute strong constraints to the acceptance of baculoviruses as insecticides.

2.3 The transcriptional regulation of the baculovirus replication cycle and the production of viral progenies

The replication cycle of baculoviruses is mainly regulated at the transcriptional level. The baculovirus transcriptional program occurs in three stages, called early, late and very late, respectively, which are coordinated in a cascade (Table 1) (Blissard & Rohrmann, 1990; Rohrmann, 2011).

Time post-infection *	RNA polymerase	Transcriptional stage	DNA replication	BVs production	OVs production	OBs assembly
0 – 8 hs.	Cellular RNA polymerase II	Early	-	-	-	-
8 – 18 hs.	Viral RNA polymerase	Late	+++	+++	-	-
From 18 hs.	Viral RNA polymerase	Very late	-	+	+++	+++

Table 1. Cascade of transcriptional events during the replication of baculoviruses and temporal distribution of production of viral progenies. *AcMNPV replication.

Each transcriptional stage is characterized by the expression of a specific group of genes. Early transcription begins immediately after the parental viral DNA is naked into the nucleus. It is carried out by the cellular RNA polymerase II, and includes a set of genes whose products are trans-activators and enzymes that will then have a role in viral DNA replication. As an exception, in the early stage is also transcribed the *gp64* gene whose expression's product is the principal glycoprotein of the group I nucleopolyhedrovirus' envelope. The end of the early stage is determined by the onset of viral DNA synthesis, and involves a change in the pattern of transcription by which some of the genes initially transcribed are no longer expressed, and a new set of genes begins to be transcribed by a viral RNA polymerase, starting the late stage of transcription. The late stage involves the expression of genes whose products are proteins and glycoproteins that are part of the structure of the budded virions, which are assembled and released from the infected cell during this time. Finally, there occurs a further change in the pattern of transcription, whose most notable feature is the hyperexpression of genes whose products are proteins and glycoproteins involved in the assembly of occluded virions and occlusion bodies, such as polyhedrin and P10. The RNA polymerase involved in the very late transcription is the

same viral enzyme in charge of the late transcription. Although some BVs are produced during the very late stage, the hallmark of this period is the assembly of occlusion bodies, which extends up to the cell death.

3. Technological applications of baculoviruses

Baculoviruses are arthropod-specific pathogens, with a host range that is generally very narrow, and lack the ability to replicate and produce pathogenic effects in other animals and plants, properties that have promoted their use as safe insecticides with reduced environmental impact (Huber, 1986). In addition, the expression of very late genes in the baculovirus genome is under the control of regulatory elements with a very high promoter activity, a property that has allowed the development of one of the most widely used expression systems for the production of recombinant proteins, the baculovirus vector expression system (Luckow & Summers, 1988). Also, baculoviruses are able to penetrate into mammalian cells, although they can not replicate into them. This property permits the use of these viruses as vectors for gene delivery (Kost & Condreay, 2002). Baculoviruses also exhibit a potent immunostimulating activity in mammals, opening the possibility of their use as adjuvants in the formulation of novel vaccines (Abe & Matsuura, 2010). Some of these applications have yet to demonstrate its market potential, but others are a reality and products based on these viruses are used today in agriculture, veterinary medicine and human medicine, among others.

3.1 Baculoviruses as insecticides

Baculoviruses presents a number of advantages over traditional synthetic chemical insecticides (Moscardi, 1999). Their high specificity makes them safe for other insects, and thus helps to preserve and even enhance the natural mechanisms of plagues control. In addition, although baculoviruses can infect mammalian cells, including human cells, they can not replicate in them and therefore they lack of pathogenicity for the human being and other animals, making safe their use. The multiplication in their natural hosts, and their capacity to persist in the environment make them suitable for the inoculative control of plagues in forestry. In addition, the same properties coupled with the aforementioned preservation of natural enemies, permit the reduction of the number of the applications needed to keep the insect plague under control in annual crops, thus contributing to reduce the costs of protection. Finally, its use in replacement of synthetic insecticides helps to reduce the overall levels of chemical pollution.

The use of baculoviruses as insecticides also has limitations. Their high specificity is also a disadvantage to their widespread use, since they are only useful when the damage to the crop to be protected is produced predominantly by a single insect, and they are not effective in controlling pest complexes. The insecticidal effect of baculoviruses is not evident immediately after application, and the delay usually is accompanied by an increase of the level of crop damage. This defect can be counteracted by an earlier application of the virus, but it requires a close quantitative following of the insect population. Also, since baculovirus production processes are based on viral replication in living hosts, their yields can not match the high yields obtained at relatively low costs in the synthesis of chemical insecticides. Table 2 presents a list of selected baculoviruses belonging to the genus *a-baculovirus* registered for their use as insecticides. As can be

observed, most of them are rather specific for one lepidopteran specie, except AcMNPV that has a wider host range.

Virus	Insect target	Crops
AgMNPV	<i>Anticarsia gemmatalis</i>	Soybean
AcMNPV	<i>Autographa californica</i> , <i>Trichoplusia ni</i> , <i>Pseudaletia includens</i> , etc.	Cotton, cabbage, tomato, broccoli
HzSNPV	<i>Helicoverpa zea</i> , <i>Heliothis virescens</i>	Cotton, corn, tomato, vegetables
HaNPV	<i>Helicoverpa armigera</i>	Cotton, tomato
LdMNPV	<i>Lymantria dispar</i>	Forest
MbMNPV	<i>Mamestra brassicae</i>	Cabbage
OpMNPV	<i>Orgyia pseudotsugata</i>	Forest
SeMNPV	<i>Spodoptera exigua</i>	Vegetables
SINPV	<i>Spodoptera littoralis</i>	Cotton
SpltMNPV	<i>Spodoptera litura</i>	Vegetables, cotton

Table 2. Examples of *a-baculovirus* registered for insecticidal use.

3.2 Genetically modified baculoviruses as improved insecticides

As explained above, the adoption of baculoviruses as insecticides is limited by some of its pathogenic properties. One of the strategies developed to overcome this limitation is the modification of the viral genome in order to improve the insecticidal capabilities of the modified virus. To this end, two alternative routes have been followed: the insertion of foreign genes whose expression gives the virus an increased virulence, and the deletion of viral genes responsible for the delay in the evolution of the viral pathogenic process (reviewed by Szewczyk et al., 2006). The genes corresponding to several specific insect toxins, hormones or enzymes have been cloned and expressed in different baculoviruses, resulting in most cases in increased virulence, decreased time to insect death, and decreased plant damage. Besides, the deletion of the viral gene codifying for the ecdysteroid glycosyl transferase (*egt*) - involved in the metabolism of the hormone ecdysone - also resulted in reduction of food consumption and faster killing of infected larvae.

Although the genetic modification has demonstrated to be a promissory strategy to improve the insecticidal ability of these viruses, the public perception about the risks that would involve the field release of recombinant viruses has limited the interest in developing novel insecticides based on genetically modified baculoviruses. In fact, although have elapsed 20 years since the first publications that described the development of genetically modified baculovirus with enhanced insecticidal activity, no one product based on these recombinant viruses has yet come to market, and companies that were involved initially in these developments have canceled the processes for obtaining approvals for its use.

4. Insect cell culture technology

4.1 A brief history

The first studies conducted *in vitro* on tissues of invertebrate animals were made by Goldschmidt in 1915 (Day & Grace, 1959). Thereafter, and for about 40 years, attempts to multiply insect cells and tissues *in vitro* have had limited success. After completion of the Second World War, and already having the air filtration technology that permitted the safe handling of animal cell cultures in sterile environments, the work of Wyatt et al. (1956) on the chemical composition of the insect hemolymph allowed the development of the first culture media specifically designed for the cultivation of lepidopteran cells (Grace, 1958). The establishment of the first insect cell lines obtained from tissues of lepidopteran insects was an achievement reached by Grace (1962). Since then until now, at least half thousand cell lines, from different insects and distinct tissues have been established. A milestone in this process was the establishment of the cell line IPLB-Sf21 (Vaughn et al., 1977). This cell line, used to plaque baculovirus for the first time, exhibited relevant technological properties, such as the ability to grow indistinctly in static cultures and in agitated suspension cultures. Also, IPLB-Sf21 was the insect cell line where the clone Sf9 was produced from. This clone was closely linked to the development of the baculovirus – insect cell expression system for recombinant proteins (Summers & Smith, 1983). At the same time, new culture media were developed, such as MM, TC-100, TNM-FH and IPL-41, and the insect haemolymph that was initially used to supplement them, was replaced by fetal calf serum. At the end of the 80's, two important developments opened the possibility of expanding the cultivation of insect cells to an industrial scale: first, the development of microemulsions of lipids and sterols allowed the formulation of serum-free media, and second, the demonstration of the protective effect of surfactant poli-alcohols on the integrity of insect cells in suspension cultures aerated by sparging permitted the scaling-up to large stirred tank reactors and airlift reactors (Maiorella et al, 1988). In recent years, the main contributions to the technology of cultivation of insect cells have come from the development of genetically modified cell lines, capable, for example, to produce proteins with humanized molecular structures (Shi & Jarvis, 2007).

4.2 Insect cell lines

In a process of baculovirus production in cell cultures is crucial to make a proper selection of the cell line to be used as substrate for virus multiplication. The selected cell line must be susceptible and permissive to the virus, which must replicate in abundance to produce high yields of both budded virus and occlusion bodies. Preferably, nutritional requirements and metabolism should be well characterized, and the cell line should show relevant technological properties such as adaptability to suspension cultures, capability to grow in a low-cost serum-free medium and ability to grow in industrial bioreactors. Furthermore, it should be genetically stable, and should not be a source of viral variability.

Currently there are hundreds of cell lines established from tissues and organs of lepidopteran insects (Lynn, 2007), but very few meet the requirements described above. Table 3 shows a list of the lepidopteran insect cell lines more used for producing wild-type and/or recombinant baculovirus. The IPLB-Sf21 cell line and its clone Sf9 have been used intensively and they are well characterized. They can grow in suspension cultures at high cell concentration in bioreactors and there are several serum-free media available for them. Both wild-type and recombinant AcMNPV replicate very well in Sf cell lines

(O'Reilly et al., 1994). In addition, these cell lines had shown to be susceptible and permissive to the replication of other baculoviruses (Claus et al, 1993). The cell line BTI-TN-5B1-4, known commercially as High Five®, is also being used widely to produce recombinant proteins due to its susceptibility to AcMNPV and elevated specific productivity (Chung & Shuler, 1993).

Cell line	Insect	Tissue of origin	Susceptibility to baculoviruses
IPLB-Sf21 / Sf9	<i>Spodoptera frugiperda</i>	Ovarioles	AcMNPV, AgMNPV, SfMNPV, SINPV, TnSNPV
BTI-TN-5B1-4	<i>Trichoplusia ni</i>	Embryos	AcMNPV, AgMNPV, TnSNPV
BCIRL-HZ-AM1	<i>Heliothis zea</i>	Ovarioles	AcMNPV, HaNPV, HzSNPV
Bm5	<i>Bombyx mori</i>	Ovarioles	AcMNPV, BmNPV
IPLB-LdEIta	<i>Lymantria dispar</i>	Embryos	AcMNPV, LdMNPV
saUFL-AG-286	<i>Anticarsia gemmatalis</i>	Embryos	AcMNPV, AgMNPV

Table 3. Lepidopteran insect cell lines used frequently to produce wild-type and recombinant baculoviruses.

Besides the widely used cell lines that were mentioned in the preceding paragraph, other cell lines have been used more narrowly in processes involving the multiplication of other baculoviruses. In general, these lines were established from tissues of the natural host of the baculovirus to replicate, because viral yields tend to be higher in infected cultures of these homologous cell lines than in the heterologous ones. For instance, the cell lines saUFL-AG-286 (*Anticarsia gemmatalis*) (Sieburth & Maruniak, 1988), BM5 (*Bombyx mori*) (Grace, 1967), BCIRL-HZ-AM1 (*Heliothis zea*) (McIntosh & Ignoffo, 1981) and IPLB-LdEIta (*Lymantria dispar*) (Lynn et al, 1988) have been used to produce specifically the viruses AgMNPV, BmNPV, HaSNPV and LdMNPV, respectively. However, these cell lines are not as well characterized as the most widely used lines, and their technological properties (adaptation to suspension, ability to grow in serum-free culture media) are less remarkable or yet unknown.

4.3 Nutrition and metabolism of lepidopteran insect cells

Most existing data on nutrition and metabolism of lepidopteran insect cells in culture has been obtained from studies of a few cell lines, mostly Sf9 and BTI-TN-5B1-4. The comparative analysis of that information permits to conclude that each cell line is considerably flexible for satisfying their nutritional requirements. However, there are marked differences in metabolic behavior between different cell lines. Carbohydrates and amino acids are the most important nutrients, and the knowledge about their quantitative demands and metabolism will be briefly reviewed below. The information available about

the quantitative requirements of other important nutrients, such as lipids, sterols, vitamins and mineral salts, is much scarce.

Carbohydrates are essential components of all culture media for insect cells, due to their role as main sources of carbon and energy. Insect cells are capable to grow in culture media containing glucose as the unique carbohydrate, but insect cells can also consume other monosaccharides and disaccharides (Mitsubishi, 1989). Sf9 cells consume glucose without production of lactate. This behavior has been attributed to the existence of an active tricarboxylic acid cycle, where 70 – 80% of the consumed glucose would be totally oxidized (Neermann & Wagner, 1996). However, these results are still controversial, because other studies have found that the percentage of glucose consumed that is derived to the tricarboxylic acid cycle is much lower (Benslimane *et al.*, 2005). The production of other metabolites such as alanine, glycerol and ethanol, as well as fatty acid synthesis, could explain the fate of a significant fraction of carbon incorporated to Sf9 cells through glucose consumption (Drews *et al.*, 2000; Bernal *et al.*, 2009). BTI-TN-5B1-4 and BM5 cell lines have a different behavior: both cell lines produce lactate, displaying a behavior similar to that of mammalian cells (Rhiel *et al.*, 1997; Stavroulakis *et al.*, 1991). The flux of glucose was studied in BTI-TN-5B1-4 cells, resulting that the proportion oxidized in the tricarboxylic acids cycle was lower than in Sf9 cells (Benslimane *et al.*, 2005). The influence of baculovirus infection on the metabolism of insect cells, and specifically on glucose metabolism, is a topic that has been scarcely addressed, and the information is contradictory (Bernal *et al.*, 2009; Gioria *et al.*, 2006).

According to Mitsubishi (1989), 15 amino acids are essential to insect cells. Glutamine is the amino acid consumed faster and in a greater extension in cultures of most lepidopteran insect cell lines characterized to date. However, it has been demonstrated that glutamine is dispensable for the growth of Sf9 cells, providing that the cells have ammonium as nitrogen source (Öhman *et al.*, 1996). Other amino acids that can be consumed in significant quantities are asparagine, aspartate, glutamate and serine, and precisely asparagine is consumed faster than glutamine in cultures of the BTI-TN-5B1-4 cell line (Rhiel *et al.*, 1997). The demand of other amino acids is much lower. Besides serving as precursors for the synthesis of proteins and nucleic acids, amino acids that are consumed faster are used as sources of energy, such as glutamine and asparagine. The metabolism of glutamine has been studied in Sf9 cells, where the utilization pathways are depending on the availability of glucose (Drews *et al.*, 2000). It has been proposed that, in glucose excess, the cytoplasmatic enzyme glutamate synthase transfers the amidic nitrogen of glutamine to the amine-position in glutamate, from where it is transaminated to alanine, the main product of glutamine metabolism. But when glucose is exhausted, glutamine is metabolized in mitochondria, where the amide-nitrogen and the amine-nitrogen of glutamine are sequentially released as ammonium ion, which accumulates as the main product of glutamine metabolism under glucose limitation in Sf9 cells. The metabolism of glutamine and other amino acids used as energy sources in other cell lines, such as BTI-TN-5B1-4 and saUFL-AG-286, is probably regulated differently, because they produce ammonia even in the presence of excess glucose (Gioria *et al.*, 2006; Rhiel *et al.*, 1997). The information about the influence of baculovirus infection on glutamine requirement and metabolism is scarce, but most results appear to indicate that insect cells tend to reduce the demand after infection (Gioria *et al.*, 2006; Bernal *et al.*, 2009).

4.4 Culture media for lepidopteran insect cells

Almost all media used for cultivation of insect cells have a chemical composition partially defined. They consist of a basal medium composed of chemically defined mixtures of carbohydrates, amino acids, vitamins, salts and, in some cases, organic acids. In addition, the media must be supplemented with compounds of undefined chemical composition that contribute to cell's proliferation, such as fetal calf serum, microbial extracts and/or protein hydrolysates.

4.4.1 Basal culture media

TC-100, IPL-41, Grace and TNM-FH are the most commonly used basal media for *in vitro* culture of lepidopteran insect cells (Schlaeger, 1996). TC-100 and TNM-FH have almost the same amino acids composition as the Grace medium, from which they originated. IPL-41 has the same qualitative composition of amino acids, but with higher concentration for the ones consumed faster: glutamine, asparagine, glutamic acid, aspartic acid and cystine. In any case, it has been demonstrated for several different insect cell lines that the concentrations of most amino acids in basal culture media are oversized with respect to cellular requirements (Bédard et al., 1993; Ferrance et al., 1993; Lua & Reid, 2003). TC-100 and TNM-FH contain also protein hydrolysates, and the last is additionally supplemented with yeast extract. With regard to carbohydrates, all media contain glucose, although at different concentrations, and IPL-41 contains also an additional monosaccharide, fructose. Grace, TNM-FH and IPL-41 media also contain disaccharides, all of them contain sucrose and the last also maltose. IPL-4 is richer in vitamins and also contains organic acids, not present in the original formulations of the media Grace, TNM-FH and TC-100. The composition of mineral salts of IPL-41 differs from the other three, and is also enriched with oligoelements. In spite of the differences in the chemical composition described above, the four basal media can support the growth of Sf9 and BTI-TN-5B1-4 cell cultures, provided they are supplemented with fetal calf serum. This fact highlights the nutritional plasticity of these cell lines. On the other hand, the richer medium IPL-41 has demonstrated to be better suited than the other three to formulate serum-free media (Ikonomou et al., 2001; Maiorella et al., 1988).

4.4.2 Fetal calf serum

Blood serum obtained from bovine fetuses is the most used undefined supplement for animal cell cultures, including both mammalian and insect cells. It concentrates, in a single component, several essential functions for cultured cells (Barnes & Sato, 1980). Serum provides, in a water-soluble vehicle, lipids and cholesterol, and it is also a rich source of growth factors, vitamins and mineral oligoelements. Its proteins' transporters allow the supply of poorly soluble ions, such as Fe⁺⁺⁺. In addition, serum has detoxifying activity, and their proteins can contribute to the preservation of the structural integrity of cells when they are subjected to mechanical stress.

In spite of the advantages of fetal calf serum, they are accompanied by several disadvantages. Its composition is undefined and variable from batch to batch. It is also a possible vehicle for the introduction of chemical and biological contaminants, such as plaguicides, virus and prions, among others. The high concentration of serum proteins may involve interference with the extraction and purification of products. Besides, serum proteins are a source of foam in culture processes where aeration is made by sparging. The cost of using fetal bovine serum as a supplement of a basic medium is so high that it can

reach 90% of the final cost of complete culture medium, which can be unacceptable for the development of a production process for a viral bioinsecticide. Finally, the regulatory agencies are becoming increasingly restrictive in relation to the use of raw materials of animal origin.

4.4.2.1 Serum-free media for insect cell cultures

The problems about the use of serum as a supplement in animal cell cultures, described above, have driven the development of new culture media capable to support the growth of insect cell cultures in a serum-free environment. As the serum is a very complex substance, and its functions are diverse, it is necessary to use a mixture of various components to replace it. The supply of hydrophobic nutrients is replaced by adding microemulsions that contain a source of lipids and cholesterol (Maiorella et al, 1988; Ikonomou et al., 2001). The most used sources of lipids are the methyl esters of fatty acids isolated from the liver of marine fishes, but recently it has been described the use of cooking soybean oil, a cheaper and more abundant source of lipids (Micheloud et al., 2009). In addition, microemulsions also contain the surfactant Pluronic F68, whose presence protects the cells from the detrimental influence of bubbles in sparged bioreactors.

On the other hand, the contribution of growth factors that made the serum is replaced by mixtures of enzymatic hydrolysates of proteins and yeast extract (Schlaeger, 1996). Hydrolysates from milk proteins such as lactalbumin and casein, and meat proteins, are commonly used as cheap replacements of serum, but the peptides responsible for the growth factor activity have not been identified. The effects of the addition of yeast extract to culture media for insect cells are similar to that of fetal calf serum, modifying the specific growth rate and increasing the maximum cell density (Eriksson & Häggström, 2005).

There are currently several commercial serum-free media available that were specifically designed to cultivate either Sf9 or BTI-TN-5B1-4 cell lines, but eventually can also support the growth of other insect cell lines. The growth parameters of cultures in these media are remarkable, as well as the yields of baculovirus and recombinant proteins obtained in infected cultures. However, the cost of the commercial serum-free media is, at least, as high as the cost of complete medium supplemented with fetal calf serum, precluding its utilization for the economically feasible production of insecticidal baculoviruses.

New serum-free media were specifically designed in recent years for culturing a few cell lines of interest, due to their potential application to the development of production processes for insecticidal baculoviruses in insect cell cultures. A prototype low cost medium (LCM) was developed to both cultivate the cell line BCIRL-HZ-AM1 and to produce the baculovirus HaSNPV (Lua & Reid, 2003). Comparable maximum cell densities and growth rates were obtained in both the LCM and a commercial serum-free medium, but lower specific virus yields were reached in LCM. The composition of the LCM medium was not disclosed. On the other hand, the low-cost medium UNL-10 was developed to grow the saUFL-AG-286 cell line, useful to produce the baculovirus AgMNPV (Micheloud et al, 2009). The yields of occlusion bodies in suspension cultures, using optimized parameters of infection, were as high as 3×10^{11} OBs L⁻¹, with specific yields higher than 600 OBs/cell. The composition of the UNL-10 medium, that was optimized to improve the yield of OBs of AgMNPV, has glucose as the only source of carbohydrates, a lower concentration of most amino acids, an improved mixture of vitamins and a lipid emulsion made with cooking oil. The growth factor activity is exerted by an optimized mixture of an enzymatic hydrolysate of casein, tryptose broth and yeast extract.

4.5 Physicochemical conditions in insect cell cultures

The pH of the medium in insect cell cultures is determined by its chemical composition, depending mainly on the buffer activity of salts, although the mixture of amino acids can also play a role in pH regulation. The optimal pH for all lepidopteran insect cell cultures is acid, between 6.2 and 6.4 (Schlaeger, 1996). While the pH can be modified through the evolution of an insect cell culture, the changes tend to be limited and usually do not compromise the cellular physiology.

The optimum osmolarity can differ for distinct lepidopteran insect cell lines, which can react also differently according to the agents utilized to modify it. The osmolarity of most culture media for insect cells varies between 250 and 350 mOsm kg⁻¹, but the initial value is habitually modified through the evolution of the culture, usually without consequences on the cellular physiology (Kurtti & Munderloh, 1984).

Insect cells are cultivated *in vitro* at temperatures ranging between 25 and 30 °C. The optimum temperature for most lepidopteran cell lines is 28 °C (O'Reilly et al., 1994). Cells cultured in serum-free media are less tolerant of temperature changes than cells grown in media supplemented with serum (Mitsubishi & Goodwin, 1989).

The dissolved oxygen concentration is a critical parameter in insect cell cultures due to the reduced solubility of oxygen in aqueous culture media (Palomares & Ramírez, 1996). Dissolved oxygen levels between 40 and 70% are usually appropriate to keep acceptable growth parameters in insect cell cultures. In addition, most of the available information indicates that it is also critical to keep a proper level of dissolved oxygen in baculovirus-infected cultures, because oxygen deprivation is a cause of low yield of virus or recombinant protein.

5. Bioreactors in insect cell cultures

Although the most used insect cell lines can grow in suspension or as static cultures indistinctly, the scaling-up of static cultures is not a feasible alternative for the production of insecticidal baculoviruses. Thus, in this section it will only be reviewed the information about insect cell suspension cultures, from agitated erlenmeyers and spinner-flasks for low-scale cultures up to stirred tank and airlift reactors at larger scale.

The suspension cell cultures at low scale in agitated erlenmeyers or spinner-flasks usually do not offer significant technological difficulties, being able to reach cell densities as high as 1 x 10⁷ viable cells per milliliter, with doubling times from 18 to 30 hours during the exponential growth phase (Bédard et al., 1993; Benslimane et al., 2007; Gioria et al., 2006; Lua & Reid, 2003; Rhiel et al., 1997). This is true for most insect cell lines as long as the ratio between the areas of the gas phase –usually air– to the liquid phase –culture medium– is large enough to ensure that the superficial supply of oxygen is adequate. In practical terms this means that the volume of culture should never exceed 25% of the total volume of the container. In addition, the stirring speed should be adjusted to 60 - 80 rpm in spinner-flasks and 100 - 120 rpm in flasks with orbital shaking.

The large scale culture of animal cells in suspension requires an adequate mixing through agitation, either mechanical or pneumatic, in order to keep cells in suspension, as well as to ensure physicochemical homogeneity and adequate mass transference. But these requirements of scaling collide with certain morphological characteristics of the insect cells, as their large size and lack of cell wall, that make them fragile and sensitive to the effects of agitation and gas sparging (Trinh et al., 1994). A successful scaling-up of suspension

cultures of insect cells will be always the result of a compromise between the satisfaction of an adequate mixing and the preservation of the structural and functional cellular integrity. Stirred tank reactors, where agitation is performed mechanically through impellers, have demonstrated to be useful to cultivate insect cells at large scale (Maranga et al., 2004). The agitation rate should be carefully controlled in stirred reactors, especially when cultures are aerated by sparging, due to deleterious effects on cell functionality and viability that occurs when cultures are stirred at speeds over 300 rpm with Rushton turbines (Cruz et al., 1998). This is because the shear in the zone near to the impeller - where the energy of agitation is introduced into the reactor- is very high. Three different ways to aerate insect cell cultures in stirred tank reactors have been used: surface aeration (Kamen et al., 1991), bubble-free aeration (Chico & Jáger, 2000) and sparging (Cruz et al., 1998). Without a doubt, the aeration method that offers a better chance of escalation in stirred reactors is through direct gas bubbling. Two process parameters should be carefully considered in stirred tank reactors aerated through sparging in order to keep a proper cellular viability and functionality: the aeration rate and the bubble size. High aeration rates, as well as low bubble size are main causes of impaired growth and functionality in insect cell cultures (Trinh et al., 1994).

Airlift reactors have been much less used than stirred tank reactors, in spite they offer advantageous characteristics to cultivate large and fragile cells, as insect cells. One of the advantages of airlift reactors, when compared to classical stirred tank reactors is that they have the potential to provide high mass transfer rates with low and homogeneously distributed shear. Thus, adequate oxygen transfer rates from the gas to the liquid phase and from this one to the suspended cells can be obtained within a homogeneous environment and with a reduced exposure to sources of mechanical stress (Merchuk, 1991). On the other hand, these reactors improve their performance as it increases in size, provided that the optimal relations between the reactor geometrical parameters are preserved. In addition, due to its simplicity of design and construction, airlift reactors require less capital investment, and its operation and maintenance costs are also lower than in stirred tank reactors. While some articles have been published on the cultivation of insect cell lines in airlift reactor and its application to the production of baculovirus and recombinant proteins, there are few systematic studies for optimization of the geometrical parameters and operation in processes involving these reactors, nor models for their scaling-up (King et al., 1992; Maiorella et al., 1988; Visnovsky et al.; 2003).

In addition to the classical airlift and stirred tank reactors, other reactor designs were applied successfully to the cultivation of lepidopteran insect cells, such as the rotating-wall vessel (Cowger et al., 1999). However, given the level of production scale that should be achieved for the development of economically feasible processes, only stirred tank and airlift reactors appear to have the potential to be used for the large scale production of insecticidal baculoviruses in insect cell cultures. This is also because of the experience existing in the scale-up of cultures of microorganisms and animal cells in these reactors.

6. Baculovirus infection in lepidopteran insect cell cultures

Figure 3 outlines an infection process of infection with baculovirus in an insect cell culture. The preceding sections have addressed the characteristics of the cells, culture media and reactors. This section will deal with those aspects of the process that relate specifically to infection: the viral inoculums, the parameters of infection, the operation strategy and the product.

6.1 BVs: the viral inoculums in insect cell cultures

The baculovirus inoculum is composed by BVs that are added to the insect cell culture at the time of infection. The quality of the seed virus is a critical factor to determine the quality of the final product, the occlusion bodies, as well as the process productivity. First, the selected strain of virus should be virulent for the insect to be controlled. It should be also capable to replicate in the cell culture, yielding a high productivity of OBs. In addition, OBs produced from that inoculums should have a high biological activity. To meet these requisites, the viral inoculums should be free of genomic variants capable to reduce either the product yield or the biological activity of OBs. Two main types of genomic variants of baculovirus capable to reduce the OBs yield and biological activity have been described: the “few polyhedra” (FP) phenotype and the defective interfering particles (DIPs).

The mutations responsible for the FP phenotype, often associated with the inactivation of the gene 25k, are expressed through the following features: reduced yield of OBs, reduced content of OVs per occlusion body, reduced biological activity of OBs and increased yield of BVs (Beames & Summers, 1989; Harrison & Summers, 1995). The emergence of the FP phenotype is responsible for a sharp drop of the final yields of OBs, as well as its biological activity (Lua et al., 2002). Once emerged, the population of FP mutants tends to enrich through successive passages in insect cell cultures, due to its increased capability to produce BVs.

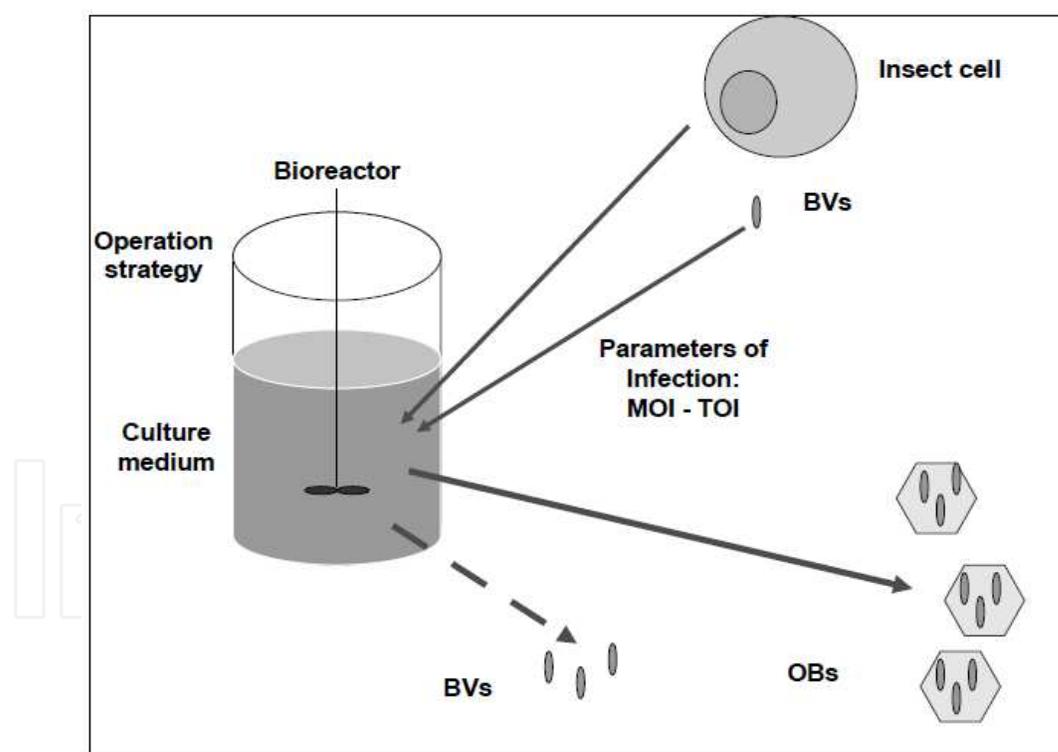


Fig. 3. Schematic representation of OBs insecticidal baculovirus production process in insect cell cultures. MOI: multiplicity of infection; TOI: time of infection.

The DIPs are generated as consequence of genomic deletions that originate shorter viral genomes (Kool et al., 1990). The DIP genomes can not replicate autonomously, but they can do it with the help of complete genomes. The replication of the DIPs competes and interferes with the replication of complete genomes, and leads to progressive enrichment of the

population of defective virus, at the expense of the population of wild-type virus (van Lier et al., 1990). The generation of DIPs in cell cultures infected with baculovirus is favored by conditions that increase the probability of homologous recombination, such as in infections at high multiplicity of infection. The proportion of DIPs in a viral population, as well as the proportion of FP mutants, increases with the number of passages in cell cultures, a phenomenon known as “passage effect” (Krell, 1996). This effect impairs the amplification of baculovirus stocks necessary to infect large scale cultures of insect cells.

The viral stock is the most expensive raw material in processes destined to the production of insecticidal OBs in insect cell cultures. Obtaining the stock for infecting the production reactor usually requires the amplification of the seed virus through successive rounds of infection in insect cell cultures at progressively larger scales (Rhodes, 1996). The optimization of the conditions of infection for the production of BVs would have a double beneficial effect on the whole production process: on the one side, it would allow to reduce the consumption of an expensive raw material and, on the other side, it would permit to reduce the steps of the seed virus amplification process, and therefore to reduce the probabilities to generate and propagate deleterious genomic variants. Despite its importance, the information available about the optimization of infection conditions for the production of the BV progeny of baculovirus is very scarce (Carinhas et al., 2009).

The quality of baculovirus stocks may also be affected by reduced infectivity in relation to the total amount of viral particles (Dee & Shuler, 1997). Although several causes have been proposed to explain this phenomenon, the inactivation of BVs could be an important detrimental factor, especially in stocks prepared in serum-free media (Jorio et al., 2006). An optimized management of the preservation of serum-free baculovirus stocks could have an important impact on the feasibility of the scaling-up process.

6.2 Parameters of infection

The fate of a baculovirus infection in an insect cell culture is strongly dependent on the selection of the multiplicity of the infection (MOI) and the time of infection (TOI) (Carinhas et al., 2009; Licari & Bailey, 1991; Micheloud et al., 2009). MOI is defined as the ratio between the number of infectious viral particles and the number of cells of the infected culture. The selection of the MOI determines the proportion of cells that become initially infected, as well as the distribution of infectious particles per cell. In cultures infected at high MOI (greater than 5 infectious units per cell), all the cells are infected synchronously. This prompts the viral replicative process to follow the same temporal pattern in all cells, with the emergence of a unique peak of viral progeny. On the other hand, when cultures are infected at lower values of MOI, only a proportion of the cellular population is infected initially. Thus, at least two cellular sub-populations will coexist in a culture infected at low MOI: infected and uninfected cells. Viral replication takes place immediately in initially infected cells, producing a viral progeny after one generation time. Uninfected cells, on the other hand, proliferate and will be infected later with the viral progeny of the initially infected cells. An important difference is that in cultures infected synchronously, viral replication occurs in cells that are in similar physiological state and subjected to similar environmental conditions, while in asynchronously infected ones viral replication takes place through successive rounds of infection, in cells under different physiological states and subjected to different environmental conditions. The importance of this difference is that the replicative capacity of insect cells varies, depending on both their physiological condition and the quality of the culture medium at the time that they are actually infected.

One factor that has contributed strongly to limiting the development of feasible production processes of insecticidal baculoviruses in insect cell cultures is the so called “cell density effect” (Wood et al., 1982). This phenomenon is characterized by the reduction of the intrinsic ability of insect cells to replicate baculovirus as the infection is delayed in time, and the cell density increases. The phenomenon has been observed also for recombinant proteins produced by genetically modified baculovirus. Several causes have been proposed to explain the “cell density effect”, including nutrient limitation (Bédard et al., 1994), accumulation of toxic by-products (Taticek & Shuler, 1997), autocrine factors and cell cycle distribution (Braunagel et al., 1998; Calles et al., 2006), and inhibition of the central energy metabolism (Bernal et al., 2009), among others, but the causes remain to be identified. The cell density effect, that could be observed in several insect cell lines infected with different baculoviruses, both wild-type and recombinant, can be overcome, at least partially in Sf9 cell cultures by adopting alternative strategies for cell culture and viral infection, such as fed-batch or perfusion, as will be explained hereinafter.

6.3 Operation strategy

The typical strategy for the production of viruses in cultured animal cells is the infection of batch cultures. This strategy implies that a cell culture is cultivated in a proper medium up to reach the desired cellular concentration, when it is added the viral inoculum and the infection is allowed to progress without ulterior modification of the system until the harvest of the product is made. The batch production of insecticidal baculoviruses can be easily implemented, and it is possible to obtain high yields of occlusion bodies (Lua & Reid, 2003; Micheloud et al., 2009; Rodas et al., 2005). However, the cell density effect is a strong limitation to reach economically significant yields of virus, so that the MOI and the time of infection should be optimized to avoid an increase in cell density of infected cultures. This limitation could be partially overcome through the medium exchange before infection (Bédard et al., 2004). Although this strategy is feasible for working at low scale in shaken-flasks or spinner-flasks, it is difficult to implement at larger scale.

Fed-batch is an alternative operation strategy to overcome the cell density effect in cultures of the *Spodoptera frugiperda* cell lines. Concentrated solutions containing the more demanded nutrients (glucose, glutamine and other amino acids, yeast extract and lipids' emulsion) were added to high density cell cultures for obtaining high yields of recombinant proteins and occlusion bodies (Bédard et al., 1994; Elias et al., 2000). The main advantage of this strategy resides in its technological simplicity, which makes it proper to be implemented in both low and large scale. However, the use of this kind of operation is limited to insect cell lines that do not accumulate toxic by-products, such as IPLB-Sf21 and Sf9. For other, cell lines, like BTI-TN-5B1-4 and saUFL-AG-286, fed-batch would not be a suitable strategy, since ammonia accumulation could be the cause of inhibition of viral replication.

The perfusion strategy, that implies the continuous removal of spent medium and its replacement by fresh medium with retention of the cell mass inside the cultivation device, has been also used. It has been employed to obtain high yields of recombinant proteins in insect cell cultures of high density, infected with genetically modified baculoviruses (Chico & Jäger, 2000). The advantage of this strategy with regard to fed-batch resides in the possibility to use it with cell lines that accumulate toxic by-products, which are continuously removed with the spent medium. However, its implementation requires sophisticated devices to remove spent cell-free medium, making it unlikely use in the development of economically feasible processes for the production of insecticidal baculoviruses in large scale.

The production in culture systems that operate continuously appears to be an attractive option to reduce the operation costs in fermentation processes. Given its lytic nature, a continuous process for baculovirus production should involve the use of at least two cultivation devices, one for cellular propagation and a second one for virus infection and replication. The first reactor is continuously fed with fresh medium, and the viral product is continuously harvested from the second reactor, which is in turn fed with cell culture from the first reactor, also in a continuous way and at the same rate. Although it has been demonstrated that is possible to operate this kind of continuous process to produce baculovirus OBs by a limited time, the viral yield is affected rapidly by the passage effect, invalidating this strategy as an effective approach (van Lier et al, 1990).

6.4 Product yield and quality

The feasibility of a production process for an insecticidal baculovirus will finally reside on the yield and the quality of viral occlusion bodies. Rhodes (1996), through a detailed economic analysis of an *in vitro* process for production of an insecticidal baculovirus, has established that the limit yield to reach the economical feasibility should be at least 2×10^{11} OBs.L⁻¹. More recently, Nguyen et al. (2011) have argued that the minimum volumetric yield for an economically feasible process of production of HaSNPV in insect cell cultures should be 40 times higher. The OBs yields effectively reached for several baculoviruses in serum-free cultures of different cell lines, according to the available information, ranged from 1×10^9 to 3×10^{11} OBsL⁻¹, with cell specific yields ranging from 3 to 700 OBs per cell (Chakraborty et al., 1999; Gioria et al., 2006; Lua & Reid, 2003; McKenna et al., 1997; Micheloud et al., 2009; Rodas et al., 2005).

The insecticidal potency of baculovirus occlusion bodies that are produced in insect cell cultures is a controversial topic. Some papers have shown that occlusion bodies produced in infected insect cell cultures are less potent than polyhedra produced by infection of insect larvae (Chakraborty et al., 1999; McKenna et al., 1997). However, this reduced activity may not be an intrinsic characteristic of the occlusion bodies produced in cell cultures, but related to the extraction method used. Extraction with a solution of sodium dodecyl sulfate (SDS), a widely used procedure for releasing the occlusion bodies accumulated in the nuclei of infected cells, alters the structure of the polyhedron envelope and would reduce the content of occluded virus per polyhedron, and thus its insecticidal ability (Lua et al., 2003). Consequently, the extraction with SDS should be avoided for the purpose of preserving the quality of occlusion bodies. On the other hand, the occlusion bodies produced by infection in larvae contain an alkaline protease that is not codified in the viral genome, and that would be incorporated from the tissues of the infected insect. This protease, which is not present in the occlusion bodies produced in cell cultures, could be an additional factor of virulence, accelerating the dissolution of occlusion bodies in the insect midgut and thus contributing to increase its biological activity (Rohrmann, 2011). Finally, it has been also reported that the composition of the culture medium could affect the activity of occlusion bodies, but the causes are unknown (Pedrini et al, 2006).

7. Scaling-up: limitations and possibilities

Although the production of insecticidal baculoviruses in insect cell cultures has been proposed as an alternative to overcome the limitations of the processes *in vivo*, so far no process *in vitro* could be even implemented on an industrial scale, and occlusion bodies are

still produced in infected insect larvae. Some factors that 25 years ago have hindered the development of large-scale production processes for insecticidal baculoviruses in insect cell cultures, such as the sensitivity of insect cells to the stresses linked to the mechanical agitation in stirred tank reactors and to the bubble rupture in sparged bioreactors, have been resolved and several cell lines can be cultivated today in industrial bioreactors of large volume to produce occlusion bodies or recombinant proteins. However, other factors that still limit the development of feasible processes have not yet been satisfactorily resolved, and will be reviewed below.

Obtaining a cell line with relevant technological properties and with the ability to replicate the virus at a high yield of OBs, is a requirement to develop a feasible process for the production of an insecticidal baculovirus in insect cell cultures. Besides Sf9 and BTI-TN-5B1-4, there are few cell lines that fulfill these requisites. The cell line BCIRL-HZ-AM1, used to produce HaSNPV, is capable to grow in suspension cultures in a low-cost serum-free medium in stirred tank reactors. BCIRL-HZ-AM1 cells can produce high specific yields of HaSNPV OBs in infected cultures (Lua & Reid, 2003), but its ability to produce high yields of BVs, a property that is important for the scaling-up, is more limited (Pedrini et al., 2011). The cell line saUFL-AG-286, of election to produce AgMNPV, can generate high specific yields of OBs in serum-free suspension cultures, but the production of OBs is strongly inhibited at cell densities higher 8×10^5 cells mL^{-1} , thus limiting the possibility to reach very high volumetric yields of OBs (Micheloud et al., 2009). As these cell lines are heterogeneous, the isolation of cell clones with improved ability to produce baculovirus OBs appears to be a reliable possibility to enhance the productivity of viral insecticides (Nguyen et al., 2011; Pasumathy & Murhammer, 1994). For the production of other insecticidal baculoviruses will be necessary to establish new cell lines, obtained preferably from tissues of their respective target insects.

Another requisite that must be resolved before confronting the scaling-up of an insecticide baculovirus production process is the development of a low cost serum-free culture medium for the selected cell line. It has been indicated that the cost of the culture medium for an economically feasible process should not be higher than US\$ 2.5 per liter (Rhodes et al., 1996), or it even should be lower than US\$ 1 (Gong et al., 1997). Commercial serum-free media for Sf9 and BTI-TN-5B1-4 cells are sold at prices that are 30 times greater, and therefore are not useful for producing insecticidal baculoviruses at industrial scale. Besides, the cost of media specifically developed for producing insecticidal baculoviruses are yet above the acceptable limit for an economically feasible process. The rational approach to further reduce the cost of culture media for insect cell cultures is the simplification of the chemical composition, based on the deep knowledge of the nutritional demands and metabolism of insect cells, both uninfected and infected. However, most cell lines have not been sufficiently characterized as to progress towards a simplification of the composition of the culture medium. A more empirical approach to reduce the cost of the culture medium is the replacement of costly ingredients, such as amino acids and lipids, by optimized mixtures of raw materials of lower cost such as protein hydrolysates and cooking oil.

The usual strategy to produce baculovirus occlusion bodies in insect cell cultures has been the infection of batch cultures. However, the possibility to obtain high volumetric yields of viral OBs in batch cultures is impaired by the “cell density effect”. Whenever possible, the adoption of alternative strategies of infection could be a way to overcome the cell density effect and thus improve the viral productivity. The fed-batch culture, which has proven to be a feasible alternative to increase the yield of recombinant proteins and BVs in Sf9 cell

cultures at high density, could also be an alternative strategy to increase the yield of occlusion bodies. A deeper understanding of the causes that lead to the manifestations of the cell density effect could help to design more rational feeding schedules than those used to date, and thus increase the viral productivity. However, the usefulness of the fed-batch strategy is restricted to cell lines that do not accumulate toxic by-products.

A large-scale process to produce insecticidal baculovirus OBs in insect cell cultures requires the completion of successive steps of viral amplification in growing scale (Rhodes, 1996). OBs are the final product of the whole process, but BVs are the product for each of the intermediate steps of scaling. Despite the importance to improve the yield of BVs, few studies have systematically explored the optimization of the production of this viral progeny (Carinhas *et al*, 2009). The optimization of BVs production could help to reduce the number of scaling steps necessary to get the number of virions needed to feed the OBs production reactor, and therefore reduce scaling cost. Furthermore, the reduction in the number of stages of scaling would contribute to limiting the probability of emergence of unproductive viral variants, such as FP mutants and DIPs. The approach patented by Lua and Reid (2005), using occluded virions extracted from occlusion bodies as seed, could alleviate the need for viral inoculum at the beginning of the scaling-up process, but does not prevent the need to improve the yields of BVs in the later stages. Additionally, the improvement of the ratio infectious particles/total particles, through better BVs preservation, could mean significant savings in the demand for seed virus, and therefore a step towards the feasible scaling-up of the viral insecticide production process in insect cell cultures.

8. Concluding remarks and perspectives

Baculoviruses are a group of arthropod-specific pathogens which have a significant potential to be used as safe and environmentally friendly insecticides in agriculture, horticulture and forestry. The replication of baculoviruses produces two viral progenies, budded and occluded viruses. The last are included into proteinaceous structures called occlusion bodies, which display insecticidal activity when ingested by susceptible insects. The current technology to produce insecticidal occlusion bodies is based on the viral infection of susceptible insects, but an alternative technology based on the viral replication in insect cell cultures could aid to overcome some of the limitations of the former. The insect cell line, the culture medium, the bioreactor, the virus, the infection parameters and the culture strategy are elements of the insect cell culture technology that must be optimized in order to develop *in vitro* production processes for insecticidal baculoviruses. While it is now possible to grow insect cells in large-scale industrial reactors using serum-free media to produce high yields of occlusion bodies for several baculoviruses, the current technology is still insufficient to achieve economic feasibility. To do that, in the next future the efforts should be mainly orientated:

- to gain deep insight over the insect cell biology in order to identify the factors responsible of the cell density effect;
- to improve the composition and to reduce the cost of serum-free culture media for insect cell cultures;
- to increase, through the manipulation of the infection parameters in batch cultures or, whenever possible, through optimized fed-batch strategies, the volumetric yield of occlusion bodies;

- to optimize the production of budded virus in order to reduce the length and the cost of the viral inoculum scaling-up, and to minimize the risk of generating and selecting unproductive viral variants.

Only obtaining satisfactory solutions for these remaining problems will make possible to establish economically viable processes for the production of insecticidal baculoviruses in insect cell cultures on an industrial scale.

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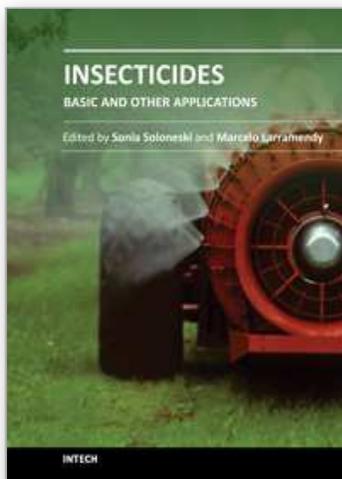
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Edited by Dr. Sonia Soloneski

ISBN 978-953-51-0007-2

Hard cover, 268 pages

Publisher InTech

Published online 08, February, 2012

Published in print edition February, 2012

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How to reference

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Juan D. Claus, Verónica V. Gioria, Gabriela A. Micheloud and Gabriel Visnovsky (2012). Production of Insecticidal Baculoviruses in Insect Cell Cultures: Potential and Limitations, *Insecticides - Basic and Other Applications*, Dr. Sonia Soloneski (Ed.), ISBN: 978-953-51-0007-2, InTech, Available from: <http://www.intechopen.com/books/insecticides-basic-and-other-applications/production-of-insecticidal-baculoviruses-in-insect-cell-cultures-potential-and-limitations>

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