Protein Production Using the Baculovirus-Insect Cell Expression System

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The baculovirus-insect cell expression system is widely used in producing recombinant proteins. This review is focused on the use of this expression system in developing bioprocesses for producing proteins of interest. The issues addressed include: the baculovirus biology and genetic manipulation to improve protein expression and quality; the suppression of proteolysis associated with the viral enzymes; the engineering of the insect cell lines for improved capability in glycosylation and folding of the expressed proteins; the impact of baculovirus on the host cell and its implications for protein production; the effects of the growth medium on metabolism of the host cell; the bioreactors and the associated operational aspects; and downstream processing of the product. All these factors strongly affect the production of recombinant proteins. The current state of knowledge is reviewed. © 2013 American Institute of Chemical Engineers *Biotechnol. Prog.*, 30:1–18, 2014
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Introduction

The baculovirus expression vector system (BEVS), first described in the early 1980s,[1] has proven successful for the production of many recombinant proteins.[2–10] In principle, any foreign gene may be expressed in the BEVS[4] for producing proteins in insect cells (IC). Protein therapeutics such as tissue plasminogen activator (tPA),[11] enzymes,[12] and viral and parasitic proteins[5,13] have been produced using the BEVS-IC. Some viral structural proteins have been shown to self-assemble into ordered structures such as virus-like particles (VLP) during production in BEVS.[14] These virus-like particles are commonly outstandingly immunogenic and therefore potentially useful as vaccines.[5,15,16] Some of the VLP vaccines have been commercialized (e.g. the human papilloma virus vaccine Cervarix^{TM})[6,8,9,17] and others are in developent.[5,7,8,17] Baculovirus expression system is promising also for delivering genes into mammalian cells for gene therapy[18,19] and other diverse applications.[20]

The production of proteins via a BEVS is a two-step process. First, the insect cells are grown to a desired concentration. In a second step, the cells are infected with a baculovirus. Like other viruses, the baculovirus takes control of the gene expression machinery of the host cell[21] and triggers responses that lead to the production of the target product. During this process, the virus of course replicates itself using the metabolic machinery of the host. Many of the consequences of the infection are well known,[22] but others are only now beginning to be understood.[23]

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From the perspective of a large-scale protein production process, the aspects to consider are the formulation of the cell culture medium, the engineering and operation of the bioreactors used in growing and infecting the cells, and the downstream processing of the crude product to a useable formulation. Here we review all these aspects of production of heterologous proteins using the BEVS. The baculovirus biology and genetic manipulation to improve protein expression and quality are discussed as are the insect cell lines for improved production of the proteins. Protein production via the BEVS-IC has been extensively reviewed,[2,4–10,24–28] but nearly always from a biological perspective. Here the focus is on the bioprocess technology of the BEVS-IC based production, but the essential biological fundamentals are discussed.

Baculovirus Biology

Baculoviriade is a large family of insect pathogenic viruses that were initially used as biopesticides to control insect pests.[29,30] Baculoviruses[31] are enveloped, rod-shaped DNA viruses that replicate in the nucleus of an insect cell. The viral particles range from 30 to 60 nm in diameter and from 250 to 300 nm in length. Baculovirus genome consists of a double-stranded covalently closed circular DNA that is 80 to 180 kbp in size, depending on the species.[32] The GenBank has records of the complete genomic sequences of at least 56 baculovirus species and isolates (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=10442). The sequence data show the presence of about 895 open reading frames encoding for structural and nonstructural proteins.[32] Thirty-one core genes have been found to be conserved in all the sequenced baculovirus genomes.[33] These genes encode proteins associated with

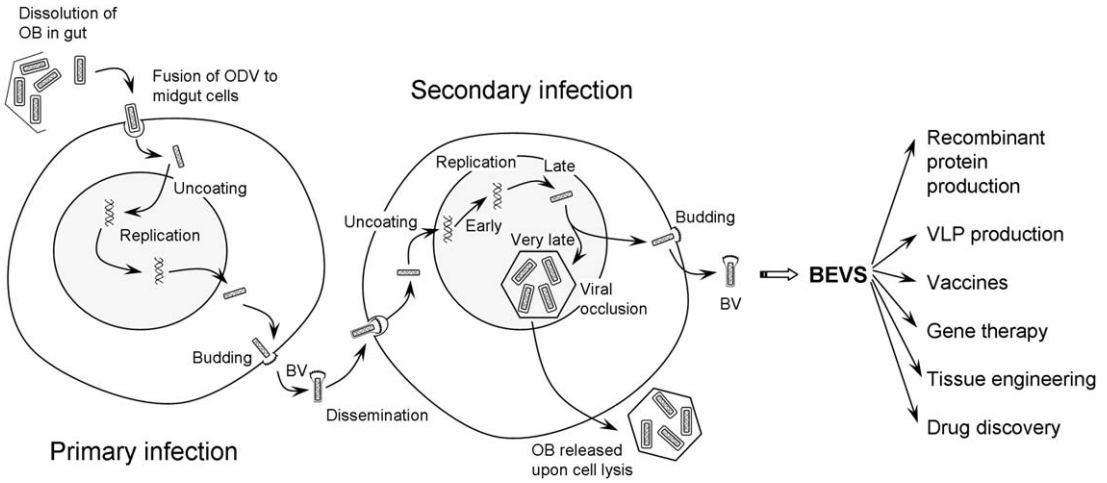

Figure 1. The replication cycle of a baculovirus. The alkaline environment of the midgut of the ingesting insect dissolves the polyhedron occlusion bodies (OBs) to release the occlusion-derived virions (ODVs). The ODVs initiate the primary infection of the insect midgut epithelial cells. The virus replicates in the midgut cells where the budded virions (BVs) form. The BVs are released from the cell via budding through the plasma membrane to infect other tissue in a secondary infection. The secondary infection results in the development of OBs which are released to the environment by cell lysis and the death of the insect, to continue horizontal transmission of infection. The proteins polyhedrin and P10 are not needed to produce BVs, the virus form associated with a systemic infection in insect cells in culture. Therefore, the polyhedrin and P10 genes in the virus can be replaced by a foreign gene to drive the expression of the target proteins. This also prevents the formation of OBs in insect cells in culture.

critical functions such as DNA replication and transcription, the virion packaging and assembly, the cell cycle arrest, and primary infection of the insect host midgut cells (oral infectivity).[4,32]

With possible exceptions, two virion phenotypes have been found in most baculoviruses. These are the occlusion-derived virions (ODVs) and the budded virions (BVs).[34] The BVs serve to spread the virus within the host whereas ODVs ensure the survival of the virus in the external environment and contribute to its spread from insect to insect.[35] ODVs are occluded in a crystalline protein matrix, the occlusion body, and set off infection in the midgut epithelium of the ingesting insect.[34] The BVs are formed after the initial infection via budding through the plasma membrane of the infected cell. They typically consist of a single nucleocapsid enveloped within a structure that is derived from the plasma membrane of the host modified by viral proteins.[34] In contrast, ODVs consist of a single or multiple nucleocapsids contained in an envelope that differs in origin and composition relative to the envelope of the BVs.[34] The replication cycle of the virus is shown in Figure 1.

Based on the different morphologies of the occluded virion particles, baculoviruses have been divided into nucleopolyhedrovirus (NPV) and granuloviruses (GV).[31,34] Both these are specific to the larval stage of their insect hosts. The NPV occlusion body (OB) contains multiple virus particles. Some of the NPVs have a single nucleocapsid within each virus particle (SNPV), while others have multiple nucleocapsids (MNPV), depending on the virus species.[4] The occlusion body diameter of NPVs ranges between 0.15 and 15 μm.[35] The NPVs are subdivided into type I and type II.[36] The type I NPVs contain the GP64 fusion protein that is required for the virus to gain entry into the host cells and for cell-to-cell transmission.[37] Type II NPVs lack the GP64 and contain instead a generic fusion protein, the F protein, that has the same role as the GP64. The F protein is found also in betabaculoviruses, deltabaculoviruses and some vertebrate viruses.[38]

The occlusion bodies (OBs) of granuloviruses (GVs) range in length from 0.15 to about 0.3 μm and usually contain a single enveloped nucleocapsid.[35] In granuloviruses, only one singly enveloped virion is occluded per inclusion body (i.e. the "capsule") of an oval shape. The major matrix protein of GVs is granulin, a polypeptide of 25 to 30 kDa that is similar to polyhedrin. The basic structure and composition of the virions of the GVs is very similar to that of the NPVs. The double-stranded, circular DNA genome of the GVs is similar in size (50–100 Da), density, and other hydrodynamic characteristics to the genomes of the NPVs.[32]

Based on phylogenetic evidence and other characteristics, a new classification system for baculoviruses recognizes four genera.[34] These are *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus*. The genus *Alphabaculovirus* includes all lepidopteran-specific nucleopolyhedroviruses, both the single nucleocapsid (SNPV) type and the multiple nucleocapsid (MNPV) type.[34] The genus *Betabaculovirus* comprises the members of the existing lepidopteran-specific genus Granulovirus. The genus *Gammabaculovirus* comprises the Hymenopteran-specific NPVs and the genus *Deltabaculovirus* is reserved for the Diptera-specific baculoviruses.[34]

Unlike in most other DNA viruses, baculovirus gene expression takes place in four phases.[4,24] These are the immediate-early phase, the delayed-early phase, the late phase and the very late phase. The immediate-early phase is associated with the expression of viral transregulators and genes that do not need transregulators for effective transcription. The genes expressed are mostly implicated in establishing the infection. The delayed-early phase is associated with the expression of genes implicated in the replication of the virus and manipulation of the host. The genes expressed in the delayed-early phase commonly require the presence of viral transregulators for efficient transcription. The late phase is associated with the production of nucleocapsids. The transition from the early to the late phase is characterized by a termination of DNA replication and protein synthesis in the

host cell. Budded viruses are produced and disseminate the infection throughout the host.

In the very late phase of gene expression, the virions become occluded and the proteins polyhedrin and P10 are produced in large amounts.[4] Polyhedrin forms the matrix of the OBs. The role of P10 is unclear, but seems to be related to the release of the OBs from the nucleus.[4,39] Viral proteases lyse the host cell and degrade the chitinous exoskeleton of the insect so that the occluded progeny virus is dispersed into the surroundings for horizontal transmission.

Baculovirus Expression Vectors

BEVS have been used extensively for the production of heterologous proteins.[5,11–13,25] Many insect cell lines are highly susceptible to infection by *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and therefore many expression vectors based on this baculovirus have been developed.[40] In fact, AcMNPV is the most widely used baculovirus vector for producing recombinant proteins in insect cells. Other baculoviruses such as *Bombyx mori* nucleopolyhedrovirus (BmNPV)[4] and *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV)[41] are used to a lesser extent than AcMNPV.

A key characteristic of these baculoviruses is their ability to produce occlusion bodies (OBs) made of polyhedrin. Polyhedrin and also P10 are the proteins involved in the horizontal transmission of infection in a larval population, but are not needed to produce budded virions (BVs), the virus form associated with a systemic infection of the host and the insect cells in culture.[4] Therefore, the polyhedrin and P10 genes can be replaced by a foreign gene under the control of the very late *polh* and *p10* promoters to drive the expression of foreign proteins in insect cells in culture.[1,42] The *polh* and *p10* are strong promoters that allow high productivities of the recombinant protein to be achieved only in the very late stage of the infection.

A protein production process can be relatively rapidly implemented using the BEVS-IC because an engineered virus is used to infect the non-modified insect cells. The infection transforms the insect cells into protein-producing factories.

The BEVS is better than yeast and bacterial expression systems for the production of biologically active mammalian proteins.[42] This is because insect cells are eukaryotic and capable of performing protein folding that resembles closely that of the mammalian cells. Furthermore, insect cells carry out protein oligomerization and post-translational modifications (e.g. glycosylation, palmitolation, myristolation, fatty acid acylation, amidation) that are similar to those of mammalian cells.[43] Notwithstanding this, the insect protein expression and processing pathways are not necessarily equivalent to those of higher eukaryotes[25] and, therefore, the expression of the desired protein, its folding and glycosylation in insect cells require careful consideration as discussed later in this review.

Protein expression

The level of expression of a protein in the BEVS depends on the protein being expressed. In general, secreted proteins such as glycoproteins are expressed in lower levels compared with nonsecreted cytoplasmic proteins.[4] The low level of production of functional glycoproteins is ascribed to problems relating to processing and/or trafficking of the non-glycosylated protein. The production of functional proteins can be enhanced through cotransfection with baculoviruses expressing chaperone proteins that prevent the protein intermediates from following unproductive folding pathways.[44]

Addition of various DNA elements to the baculovirus has also been linked to an increased expression level of some proteins,[45,46] but a broader applicability of this strategy for enhancing protein yield remains to be assessed. Genetic modification of the insect cell can also be used to enhance the production of a target protein by a BEVS. For example, a cell genetically transformed with a polydnavirus *vankyrin* gene controlled by an immediate-early baculovirus promoter may be used.[47] Baculovirus-mediated expression of this gene prolongs the viability of insect cells and increases the production of a foreign protein.[24] Strategies for co-expression of subunits of a polyprotein using the BEVS-IC system have been described.[10]

Protein glycosylation and folding

Insect cells perform most of the posttranslational modifications that occur in mammalian cells. However, the N-linked glycosylation in insect cells results in glycoproteins having only simple oligo-mannose sugar chains[48] (Figure 2), whereas in mammalian cells it results in glycoproteins having complex sugar groups with terminal sialic acids[48–50] (Figure 2). This is a major limitation of the BEVS because N-glycans contribute to the functionality of the glycoprotein in multiple ways.[25] Differences between the desired and the actual glycosylation patterns, particularly the absence of terminal sialic acids, limit the utility of the recombinant glycoproteins. These differences affect the immunogenicity and the biological characteristics of the protein compared with the native mammalian protein.[51] Attempts to address this problem[50] have resulted in the creation of insect cell lines that express the genes for the enzymes required for producing the mammalian glycosylation patterns that were lacking in the parent insect cell line.[4,52–54] The glycoengineered insect cells coupled with BEVS can produce recombinant glycoproteins with complex terminally sialylated N-glycans[48,49] (Figure 2).

In summary, production of the target protein may be improved by cotransfection of the insect cells with baculoviruses expressing chaperone proteins and engineering of the cells to express the mammalian glycosylation enzymes and foldases. Such strategies have succeeded in enhanced production of secreted complex functional mammalian proteins in insect cells.[43] These advances notwithstanding, a fuller understand of the protein glycosylation and folding pathways is necessary to further improve the capability of BEVS.

Proteolytic activity of viral enzymes

The promoters *p10* and *polh* are the most commonly used in expressing foreign proteins via the BEVS. Both these promoters are active in the very late phase of baculovirus infection when the cells undergo lysis and release the baculovirus occlusion bodies as well as the intracellular proteases.[55] This release of proteases can result in the proteolytic degradation of the expressed recombinant protein.[55] The enzymes responsible for the lysis of the host cells are chitinase and cathepsin.[56] The expression of these viral enzymes in cultured

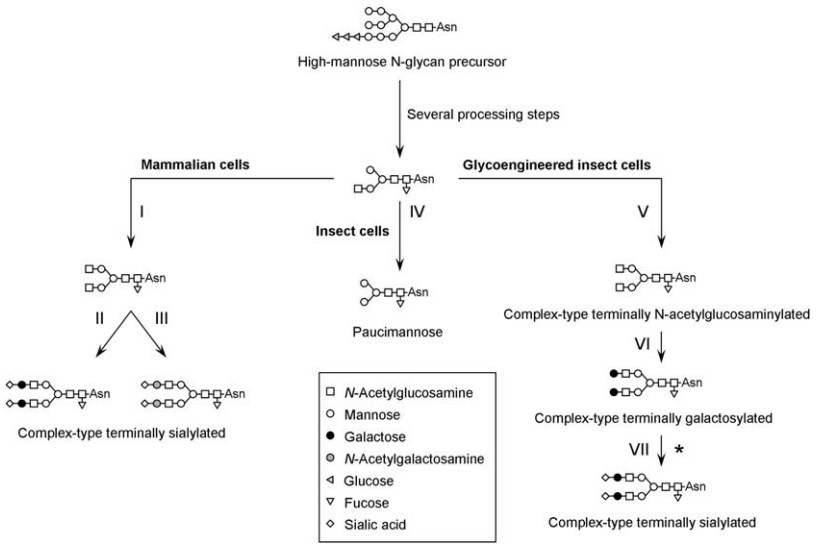

Figure 2. Recombinant protein glycosylation pathways in mammalian cells, nonengineered insect cells and glycoengineered insect cells. The N-glycan processing pathways in mammalian and insect cells begin with a common precursor and share an intermediate. In mammalian cells, the shared intermediate leads to complex-type terminally sialylated N-glycans. The latter are a diverse group and only two representative examples are shown above. Insect cells have a limited capacity for making N-glycans and produce mainly a paucimannose structure. Glycoengineered insect cells have an enhanced capability for processing N-glycans and in combination with BEVS, can produce complex-type terminally sialylated N-glycans. All known BEV-Sglycoengineered IC systems require the supplementation with acetyl-D-mannosamine, a precursor of the sialic acid, for efficient sialylation in the step marked with an asterisk (*). The enzymes involved in the various pathways are as follows: (I) *N*-acetylglucosaminyl transferase 2; (II) galactosyltransferases sialyltransferases; (III) *N*-acetylgalactosyltransferase sialyltransferases; (IV) *N*-acetylglucosaminidase; (V) alpha-1,6-mannosyl-glycoprotein 2-beta-*N*-acetylglucosaminyltransferase; (VI) beta-1,4-galactosyltransferase 1; and (VII) beta-galactoside alpha-2,6-sialyltransferase 1. Based on Harrison and Jarvis[48] and Geisler and Jarvis.[49]

insect cells is not necessary for efficient replication and high-level expression of the secreted target protein.[4] Therefore, proteolytic degradation of the product protein can be minimized by preventing lysis of the host cell. This can be done by using constructs with deleted *chiA* and *v-cath*, the genes that encode the production of chitinase and cathepsin, respectively.[57] Alternatively, the proteolysis of the product protein may be reduced by using promoters that are active in the immediate-early, the delayed-early or the late phases of the infection cycle.[26] Expressing a protein at the early stages of infection may result in more efficient processing of glycoproteins as the host cell and its secretory pathways are still in good shape.[4] Another approach to reducing proteolysis is to use a baculovirus with a poor capability for lysing the host cell.[58] Such a baculovirus isolated by random mutagenesis did in fact lead to an increased production of a target protein and less proteolysis in comparison with the parent virus.[58]

Insect cells for Baculovirus Propagation

More than 400 cell lines derived from over a 100 insect species have been used to produce baculoviruses, virus-like particles, recombinant proteins, and gene therapy vectors.[2,7,59] Insect cells typically range in diameter from 10 to 20 μm.[60] The cell morphology may be spherical or fibroblast-like.[60]

Cells of Lepidoptera insects have been traditionally the most commonly used for protein production via the BEVS. These cells were initially selected based in their potential to produce baculoviruses for use as biopesticides,[1,29,30] but are now commonly used for producing recombinant proteins via the BEVS. Instead of producing infective viruses, the focus has shifted to attaining high yields of secreted heterologous proteins and improved glycosylation patterns.[2] Insect cells have been engineered to mimic the glycosylation capability of mammalian cells. Insect cells isolated from undifferentiated ovarian and embryonic tissues are preferred for establishing cell lines for use in producing recombinant proteins. The undifferentiated embryonic tissue allows continuous diploid cell lines to be established.

Three lepidopteran cell lines are the most frequently used for protein production using the BEVSs. Two of these cell lines are derived from the pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda*. These are the IPLB-Sf21-AE cell line (Sf21)[61] and Sf9, a subclone of Sf21.[62] The third commonly used host cell line is the BTI-Tn-5B1–4, derived from the adult ovarian tissue of the cabbage looper *Trichoplusia ni*[63] and commercialized by Invitrogen as High-Five™. Other less common host cell lines in use for making recombinant proteins are: the Bm5 derived from *Bombyx mori*;[64] the Tn368 obtained from *Trichoplusia ni*;[65] and the Ea88 isolated from *Estigmene acrea*.[66]

Some of the less commonly used cell lines have the potential to produce copious amounts of recombinant proteins,[63] but they do not grow well in suspension culture and this has limited their utility. The Sf9, Sf21, HighFive™, and other lepidopteran cell lines reproduce well both in adherent cultures and suspension cultures and can be adapted to serum-free media.[25,67,68] The HighFive™ cell line is said to achieve higher titers of recombinant proteins compared with the Sf9 cells on a per cell basis.[63,69,70] However, the Sf9 cells (and also the Sf21) commonly achieve higher densities in culture than do the HighFive™ cells under the typically used culture conditions and therefore provide higher volumetric productivities than the HighFive™.[2,59]

The first genetically engineered Sf9 subcloned cell line (designated the Sfβ4GalT) became available in 1998.[71] Since then, a new generation of genetically modified insect cells have been established as expression vectors for baculoviruses, including the Super Sf9 cell lines (Oxford Expression Technologies; www.oetltd.com). The Super Sf9 cells have been engineered to express a protein that prolongs the survival of the cell postinfection and this increases the production of the heterologous protein. These transgenic cell lines, and others with humanized protein glycosylation pathways, have greatly improved the production of baculovirus-mediated recombinant proteins. Transgenic host insect cells with further improved capabilities in processing of recombinant proteins are likely to be produced.[25] Insect cell culture has been widely discussed in the literature.[26,63,72,73]

Impact of Baculovirus on the Host Cell

During infection with a baculovirus, the host cell experiences intense stress. This triggers multilevel responses within the host cell to produce biochemical and structural changes such as a rearrangement of the cytoskeleton, arrest of the cell cycle and inhibition of apoptosis.[21,22] The baculovirus takes control of gene expression machinery of the cell for its own purpose.[21,74] The utility of the BEVS as a protein factory, or a vector for gene therapy, is a direct consequence of the changes the viral infection brings about in the cell.

A baculovirus is capable of moving its genome across the nuclear membrane of the insect cells. A fusion of the virus with the cell membrane results in the release of the nucleocapsid into the cytoplasm.[22] A cytoskeletal reorganization of the cell is induced to achieve intracellular transport of the nucleocapsid to the nucleus.[75] In the nucleus, the host cell's machinery is used to synthesize viral components that assemble into new nucleocapsids.[76] The nucleocapsids migrate from the nucleus to the plasma membrane where they leave the cell through budding[77] to infect other cells. The extent of the cytoskeleton reorganization postinfection depends on the type of virus.[78,79]

In a normal insect cell, the progression from one phase of the cell cycle to the next is regulated by mechanisms that respond to the internal conditions of the cell and the external stimuli.[80] Infection with a virus results in a subversion of the cell cycle to create conditions necessary for maximizing the replication of the viral DNA.[81] This activates the cellular DNA damage response.[82,83] Baculovirus infection of insect cells leads to the cell cycle arrest in the G2/M phases and this has been found to be necessary for optimal maturation and assembly of the occlusion-derived virons (ODVs).[84] The arrest in G2/M phases is associated with a shut-off of the global host protein synthesis[85] and cessation of replication of the cellular DNA, but the replication of the viral DNA and the expression of viral genes continues.[22] The cycle phase of the cell at the time of infection appears to influence the baculovirus interaction with the host cell. For example, an insect cell in the G1 phase is most susceptible to baculovirus infection and cells in G2/M phases are the least susceptible.[23] Viral infection also activates and modulates cellular pro-survival pathways[86] to achieve an environment for optimal replication and the production of viral progeny.[87]

Apoptosis, or programed cell death, is a cellular response to prevent replication of damaged DNA. Baculoviruses have evolved mechanisms to block apoptosis in infected cells[22,88] to ensure their own replication. The P35 protein encoded by the *p35* gene present in some baculoviruses[89] has been identified as responsible for blocking the host cell's apoptotic response by inhibiting caspases.[90] In some cases, apoptosis is inhibited by the products of the *iap* gene family.[89] Nearly all the baculoviruses possess at least one *iap* gene;[89] however, the anti-apoptotic activity of *iap* genes is cell type-specific, or possibly restricted to specific scenarios.[22] In view of their widespread occurrence in viral and other genomes, the *iap* genes may have additional functions unrelated to apoptosis.[22]

Infection with a baculovirus places an additional burden on the host cells, resulting in an increased flux trough the glycolysis and tricarboxylic acid pathways.[91,92] In addition, the synthesis of viral proteins and DNA demands an enhanced biosynthetic activity of the host cell.[88] As a consequence, the oxygen uptake rate of the cells increases postinfection to generate energy via respiration.[91]

Media for Insect Cells and Cell Metabolism

Over the years, the culture media have been extensively developed. A medium formulated with the haemolymph of *Bombyx mori* moth was the first to be successfully used to culture insect cells.[93] Large quantities of haemolymph are difficult to obtain and, therefore, a basal medium was specifically designed for in vitro culture of insect cells.[94] Most cell lines of lepidopteran origin have since been grown in Grace's medium or one of its modifications. Among the media most commonly used commercially are the TNM-FH, IPL-40, IPL-41, TC-100, EX-CELL® 400, and EX-CELL® 420. Culture media typically contain a carbon source (fructose or glucose), amino acids, organic acids, vitamins, lipids and inorganic salts. The earlier media formulations were commonly supplemented with 5 to 20% (by vol) fetal bovine serum, but media free of any animal protein are now the norm. Serum and other animal proteins are potential sources of animal-derived adventitious agents and therefore are no longer used in producing parenteral protein products.[95]

One of the first serum-free medium for insect cells culture was based on the IPL-41 medium supplemented with yeastolate, lipids and Pluronic F-68.[72] Media formulations based on yeastolate have been found to support high cell concentrations and recombinant proteins titers.[96,97] Commercial serum-free media formulations include HyClone SFX-Insect (Thermo Scientific, Logan, UT), Insect-XPRESS™ (Cambrex Bio Science, Walkersville, MD, USA), ESF 921 (Expression Systems, Woodland, CA) and Express Five® and Sf-900™ II and Sf-900™ III (Invitrogen, Carlsbad, CA). Sf-900™ II has proven excellent for all stages of production, including transfection, virus preparation and amplification, and protein expression.[10] Serum-free media are typically of proprietary composition, cell line specific and expensive.[73] Serum-free media may contain plant protein hydrolysates (plant peptones)[98] to replace the functions of serum. Plant proteins pose little risk of being contaminated with viruses and pathogens of animal origin. Many insect cell lines have been grown in media containing plant peptones.[95,99,100]

Glucose is regarded as a most useful carbon source for growing insect cells,[26] but maltose and fructose can be

utilized as alternative carbon sources.[101,102] Not all insect cells grow equally well on the different useable carbon sources.[103]

Amino acids are used by the cells as precursors for the synthesis of proteins and sources of energy. The amino acid glutamine is a significant source of both carbon and energy.[101,103] Amino acids such as glutamate, asparagine, arginine, aspartate, methionine and serine are used for energy.[26] Alanine accumulates in the culture medium when glucose is in excess and is consumed once glucose is depleted.[91]

Insect cells produce lactate and ammonia during culture. Lactate is a by-product of glucose metabolism, but does not generally accumulate, except in oxygen limiting conditions.[26,104] In some cases, a low level accumulation of lactate has been reported.[105] The cell line and the composition of the medium may also affect lactate accumulation in insect cell cultures.[102]

Ammonia is a by-product of consumption of amino acids, especially glutamine, and is used up in the synthesis of new amino acids. As for lactate, ammonia accumulation is cell-line-specific and the final concentration of ammonia depends on the amount of glutamine and asparagine initially present.[102] Ammonia is produced as a result of glutaminolysis, after glucose has been exhausted and in parallel with the consumption of alanine.[101]

Culture media are typically buffered with phosphate and therefore a controlled carbon dioxide environment is not necessary for pH control.[60] The media are formulated to obtain an osmolality of around 350 mOsm kg^{-1},[60] or a little higher than the 300 mOsm kg^{-1} generally used for mammalian cell culture media.

Bioprocess Engineering Aspects of Insect Cell Culture

A major objective of a production process is to maximize the volumetric productivity of the product of interest while assuring quality. In addition to the already discussed factors related to the specifics of the BEVS used and the culture medium, the protein productivity is influenced by the type of bioreactor, the mode of operation of the bioreactor and the culture conditions.

Bioreactor systems

Many different types of bioreactor systems (e.g. stirred tanks, airlift bioreactors, wave bioreactors) have been used to culture insect cells in robust, well characterized, and cost-effective processes,[26,27,73] but stirred tank type of bioreactors are the most widely used.[59] Cells may be grown attached to solid surfaces,[60,106] but the use of suspension-adapted freely suspended cells is preferred because of its simplicity.[27] Cell concentrations of 2 to 8 $\times$ 10^6 cells mL^{-1} are readily attained in batch culture.[60] In perfusion culture, the cell concentration may reach up to around 5.5 $\times$ 10^7 cells mL^{-1}.[107] For infecting the cells with baculovirus, the maximum workable cell concentration is around 5 $\times$ 10^6 cells mL^{-1}.[60,108]

Batch and fed-batch cultures are the preferred modes of operation for large-scale production of proteins via the BEVS.[109,110] This is because batch and fed-batch operations are simple, flexible, and inherently suited to a process involving a lytic infection cycle. This notwithstanding, a batch culture is certainly not optimal for attaining a high concentration of insect cells in suspension because a progressive depletion of nutrients and accumulation of metabolites limit the maximum attainable cell concentration. Fed-batch culture overcomes some of the limitations of batch culture. Infection of a high-density culture with a baculovirus poses other problems.

Infection of a high-density culture actually leads to a reduced cell-specific production of the target protein. This phenomena is commonly referred to as the "cell density effect." Earlier studies attributed this effect to the low levels of nutrients at the instance of infection in a high-density cell culture[111,112] resulting in a low cell specific protein titer. Recent work suggests that neither the depletion of the nutrients nor accumulation of metabolites fully explain the cell density effect which may have a metabolic basis.[27,91,113,114] In principle, the cell density effect may be countered by a partial or total replacement of the culture medium prior to infection, or shortly after infection, to allow a high cell specific protein titer to be obtained.[26,104,115] A total replacement of the culture medium pre- or postinfection in a large scale operation is impractical and adversely impacts the economics of protein production.[104] A partial replacement of the medium allows the retention of the autocrine growth-promoting factors and is less expensive.[116–119] Alternatively, a part of the spent medium may be reused to provide autocrine growth factors. For example, a 20% by volume supplementation of the fresh medium with spent medium from a mid-exponential growth phase culture, has been found to substantially improve cell growth and virus production.[120]

Alternatives to total replacement of the medium include fed-batch operation,[121] semi-continuous operation in a cascade of bioreactors,[122] continuous culture[123] and perfusion culture.[124] Of these modes of operation, only the fed-batch and, possibly, perfusion culture, are practical alternatives to the batch mode of operation.

In a fed-batch culture, fresh medium is added to the bioreactor either continuously or intermittently and the culture volume increases with time. The addition of fresh nutrients may be timed to occur just prior to infection of an otherwise batch culture, or soon after infection. This prevents a total depletion of the nutrients so that the production of the recombinant protein can continue. A fed-batch operation is nearly as simple as a batch operation and much simpler than the other modes of operation that were mentioned earlier. The fed-batch operation has been extensively discussed in the literature.[105,114,122,125,126] Use of a suitably formulated feed medium and optimization of the feeding strategy have been successful in increasing the product protein titers by two to sevenfold relative to batch culture.[104,113,123,126]

In perfusion culture, the bioreactor is fed continuously and the harvest is continuous at the same volume flow rate as the feed rate, but the cells are retained within the bioreactor by some sort of retention mechanism. Although a perfusion culture is more complex than a fed-batch operation, it is being increasingly used.[109,110] In a perfusion operation with a possible periodic bleed of cells, the nutrient levels can be maintained at steady values and the product protein is continuously removed in the spent medium leaving the bioreactor. Because a perfusion operation can maintain the culture near a steady state, it has gained broad acceptance from the regulatory bodies for commercial production of recombinant proteins.[124] High cell densities are attained (e.g. 5.5 $\times$

Table 1. Modes of Operation and Feeding for Protein Production

Mode of Operation	Maximum Cell Concentration	Advantages	Disadvantages
Batch	2 to 8 $\times$ 10^6 cells/mL	Simple, flexible and inherently suited to a process involving a lytic infection cycle	Maximum cell concentration is limited by depletion of nutrients and accumulation of wastes
Fed-batch	50 $\times$ 10^6 cells/mL	Achieves higher cell densities than a batch operation	Cell-specific production of the target protein is reduced by the "cell density effect" explained in the text
Replacement of culture medium	20 $\times$ 10^6 cells/mL	Total or partial replacement of culture medium at infection counters the cell density effect and improves protein production	May adversely impact the economics of protein production because of the cost of the medium
Perfusion	55 $\times$ 10^6 cells/mL	The culture can be maintained near a steady state and the product protein is continuously removed, minimizing degradation by proteolytic enzymes	More complex than batch and fed-batch operations. Best suited for producing recombinant proteins using transformed insect cells. Not optimal for protein production via BEVS-IC processes that involve an infection phase and subsequent cell lysis

10^7 cells mL^{-1}) in perfusion culture and protein production levels can be high.[107,127,128] In addition, the residence time of the target protein in the bioreactor is minimized so that the harvested broth can be chilled, or immediately processed to reduce degradation by proteolytic enzymes.

Perfusion requires retention of the cells within the bioreactor, although periodic bleeding may be used to keep the cell concentration at some desired level. Membrane type of filters have been traditionally used for cell retention. The membrane retention device may be located within the bioreactor vessel,[107,129] or it may be placed externally.[130–132] Because of fouling and relatively low perfusion rates, membrane based retention devices have a limited potential for scale-up. In addition, the use of an external device requires the implementation of external recycle loops and pumps.[133] Because of these drawbacks, the use of highly effective ultrasonic acoustic cell separators is increasing.[127,134,135] Ultrasonic separators are effective, free of moving parts and do not foul.

Perfusion culture is eminently suited for producing recombinant proteins using transformed insect cells,[128] but is not optimal for protein production via the BEVS that involves a distinct infection phase and subsequent cell lysis.[27,136] A perfusion culture may be used to grow cells to a high concentration prior to a batch stage of infection and lysis. The common modes of culture operation are summarized in Table 1.

In small-scale operations, the use of disposable bioreactors (Figure 3) has been increasing in the production of biotechnology products for medical use.[137–139] These bioreactors are purchased as sterile ready-to-use devices that are disposed of after a single use. More than 20 different kinds of disposable bioreactors have been developed.[137] Among these, the bag type of bioreactors with wave induced mixing (Figures 3a,b) have proven especially effective for small- and medium-scale use.[138,139] Bioreactor bags with wave induced mixing are widely used in process development[110,140] and the production of inocula for large production-scale bioreactors.[137] Fairly large 2,000 L stirred tank disposable bioreactors (Figures 3c–e) are also being used commonly.[137] They have performed well[141,142] and may be superior to the traditional types of stirred tank bioreactors.[143] Single-use disposable bioreactors have been developed also for anchorage dependent cells that typically grow attached to the surfaces of microcarriers held in a packed bed, or kept freely suspended (Figure 3f).[143] Disposable bioreactors eliminate all likelihood of cross contamination of batches and greatly reduce batch failure by minimizing microbial contamination resulting from inadequate sterilization.

Culture parameters and operational conditions

A substantial effort has been made to optimize the BEVS protein production systems to maximize the productivity and enhance product quality.[41,144,145] In addition to the earlier mentioned optimization of cell density at infection and the feeding strategies, the process improvement effort has focused on all the critical factors that affect a BEVS operation. These factors include the culture temperature, the pH, the concentration of dissolved oxygen, the level of dissolved carbon dioxide, the multiplicity of infection, the time of infection, and the time of harvest (Table 2).

Insect cells are typically grown in the temperature range from 25 to 30°C.[60] The optimal growth temperature is 27 to 28°C. The temperature may be varied during a process in attempts to improve production of the product and its quality. For example, lowering the culture temperature to 20 to 24°C reduces the growth rate of the cells so that oxygen limitations can be alleviated.[146,147] This reduces the accumulation of lactate in the culture medium.[59] Increasing the temperature to 30°C enhances the rate of cell growth and consumption of nutrients (oxygen, glucose).[147] Further increasing the temperature to 35°C decreases the rates of growth and consumption of nutrients.[146] The Sf9 cells can be adapted to grow at 37°C for extended periods.[148]

The temperature affects the yield and quality of the product. A lowering of the temperature from the optimal value for growth (27°C) to 22°C has been reported to decrease the production of proteins and the amount of the product released by the cells.[146] Similarly, an increase in temperature to >27°C has also decreased the protein productivity.[146] The operating temperature has been found to significantly affect the glycosylation of recombinant proteins. For example, for the High-Five™ cell line, a lowering of the temperature from 28°C to 20–24 C results in a more complete glycosylation of the product proteins.[149] Glycosylation may be influenced by other factors, including the level of dissolved oxygen,[144] the agitation rate,[145] and the bioreactor design.[41] Both the release of proteases by baculovirus and their proteolytic activity tend to be high at the optimal culture temperature.[13] This problem requires attention in the production

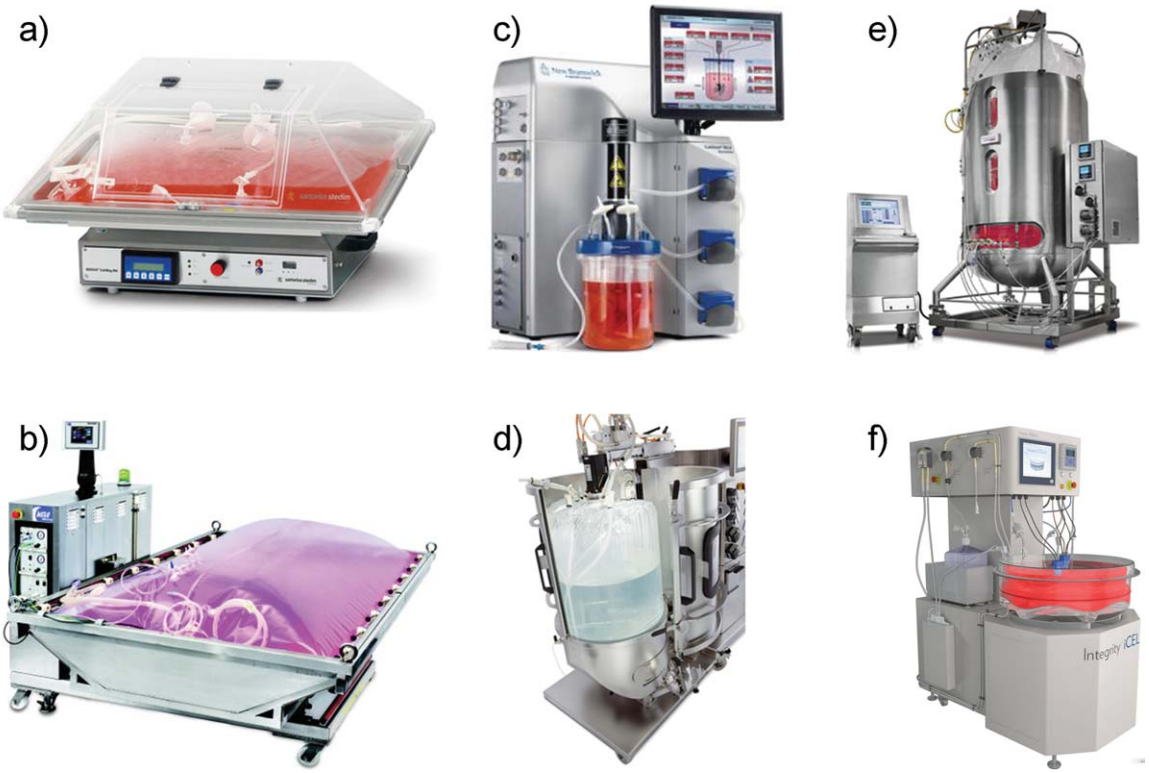

Figure 3. Some single-use disposable bioreactors for cell culture: (a) BIOSTAT® CultiBag bioreactor system (0.1–300 L) from Sartorius (www.sartorius.com); (b) WAVE bioreactor (0.1–500 L) by GE Healthcare Life Sciences (www.gelifesciences.com); (c) Celligen® BLU stirred tank bioreactor (5–50 L) by New Brunswick (www.newbrunswick.eppendorf.com); (d) BIOSTAT® STR stirred tank bioreactor (12.5–1,000 L) by Sartorius; (e) XDR stirred tank bioreactor system (10–2,000 L) by GE Healthcare Life Sciences; (f) the Integrity™ iCELLis™ single-use microcarrier-filled packed bed bioreactor for anchorage dependent cells (upto 500 m^2 growth area) offered by ATMI (www.atmi.com).

Table 2. Typical Operating Conditions of BEVS-IC Processes

Variable	Value	Observations
Temperature for growth	25–30°C	Oscillation of temperature may improve cell viability
Temperature for protein production	25–27°C	Oscillation of temperature may improve baculovirus titer. Postinfection, the temperature affects protein production and glycosylation
pH	6.2–6.4	pH may affect the entry of the virus into the cell, the replication of the virus and production of the recombinant protein
Dissolved oxygen level for growth	30–100% of air saturation	Cells are less sensitive to the dissolved oxygen concentration during growth than postinfection
Dissolved oxygen level for protein production	Often a higher level than for growth	Postinfection the rate of consumption of oxygen increases and the oxygen concentration greatly affects productivity and quality of the produced proteins
Sensitivity to shear rate	High	This limits the ability to influence the oxygen concentration by increasing the gas flow rate and the agitation intensity
Carbon dioxide level in the atmosphere	0–5% by volume in air	Accumulation of CO_2 may inhibit cell growth and protein productivity
Medium osmolality	320–375 mOsm/kg	Insect cells have a low sensitivity to variations in osmolality
Lactate accumulation	Low	Insect cells do not generally produce lactate, except in oxygen limiting conditions. However, the cells are generally highly sensitive to lactate accumulation
Ammonia accumulation	Moderate	Specific production rate of ammonia by insect cells is relatively high, but they generally have a low sensitive to ammonia toxicity
Multiplicity of infection (MOI)	Low to high	Optimal MOI depends on the cell line, the baculovirus, the medium, the mode of operation, and the physiological state of the cells. MOI affects the protein productivity, the production of defective interfering particles and the length of time the proteins are exposed to proteases

of protease sensitive products and may be overcome by using an appropriate baculovirus.

In a typical insect cell culture, the temperature remains constant, or is varied in steps. In an unusual operation involving oscillation of the temperature from 24 to 28°C, a Sf9 culture was found to have an improved cell viability and cell specific baculovirus titer compared with operation at a constant temperature.[150] A temperature shift postinfection has been reported to enhance the production of adeno-associated virus vectors.[148]

Insect cells grow at pH values in the range of 6.0 to 6.8,[151] but the optimal pH for growth is around 6.2 to 6.4

for most lines.[59] A controlled pH of 6.2 is commonly used in bioreactor culture for both the growth phase and the infection phase. While the effects of pH on production of recombinant proteins have not been investigated in detail, the pH has been suggested as a factor affecting the entry of the virus into the cell and, therefore, the replication of the virus and the production of protein are likely affected by pH to some degree.[152]

Although various values of dissolved oxygen concentration have been suggested as being optimal for growth of the different insect cell lines, in general during growth the insect cells are not particularly sensitive to variations in the dissolved oxygen concentration so long as the concentration is kept in the range of 30 to 100% of air saturation.[153,154] During infection the cells become sensitive to the dissolved oxygen concentration[13] and the rate of consumption of oxygen increases significantly.[102,155,156] The instance of the peak in oxygen consumption rate has been suggested for identifying the best time to harvest the culture.[156] The concentration of dissolved oxygen is, therefore, important in determining the protein productivity postinfection and the quality of the product.[157] The protein productivity and quality of course depend also on the cell line and the baculovirus used.

Oxygen limitation postinfection is well known to adversely affect the product expression and quality.[144,150,158,159] Preventing such a limitation by ensuring a sufficiently high rate of oxygen transfer is, therefore, important for obtaining a high productivity and protein quality. In a typical culture system, the oxygen transfer can be enhanced by increasing the oxygen partial pressure in the aeration gas, the gas flow rate and the agitation rate. There are limits to how much the gas flow rate and the intensity of agitation may be increased, as insect cells are highly sensitive to shear stress. While mechanical agitation in the absence of aeration may not damage the freely suspended animal cells,[160] aeration does promote cell damage[161] that is exacerbated by agitation. Aeration with small bubbles is commonly used to improve oxygen transfer,[162] but bubbles smaller than about 5 mm in diameter are much more harmful to cells than larger bubbles.[161,163–166] In addition, small bubbles do not disengage from the culture broth and eventually all oxygen in them is replaced by carbon dioxide generated by cell respiration. Accumulation of carbon dioxide in the culture broth may inhibit cell growth[167] and reduce productivity of the target protein.[168] The bubble-associated cell damage can be minimized by using protective additives as Pluronic F-68 and methyl cellulose.[161,166]

The multiplicity of infection (MOI) affects the protein productivity. MOI is the quantity of the virus in terms of the plaque forming units (PFUs), or infectious particles (IPs), added to the culture per insect cell at the time of infection.[169] Infection with an MOI of 1 PFU/cell, or a little higher, does not result in each insect cell receiving an infective particle. The number of virons received by each cell generally follows the Poisson distribution.[2,16,170] Thus, if an MOI of 10 is used in a culture having a total of a million identical cells, 99.5% of the cells are likely to receive more than one infective particle.

Cells can be infected using either a low or a high MOI. With a low MOI, i.e. much fewer than 1 PFUs per cell, the infection is asynchronous. Thus, only a fraction of the cells are initially infected (the primary infection) and cease to grow.[2] The uninfected cells continue to multiply. The infected cells produce new virus particles which in turn are released to infect the remaining cells in a secondary infection. A high MOI typically involves infection with >5 PFUs per cell. This is sufficient to infect all the cells simultaneously to produce a synchronous infection[16] that halts growth immediately.

The use of a low MOI is recommended for baculovirus stock amplification to prevent or minimize the production of defective interfering virons that may affect the quality of the virus inoculum.[171] Defective interfering particles have been identified as being responsible of the "passage effect," or a reduced expression capacity of the baculovirus after multiple passages.[2,171] At production-scale, a low MOI is preferred as it reduces the need for multiple virus amplification steps for inoculum development and minimizes the volume of the baculovirus inoculum.[60,172,173] A small volume of inoculum reduces the amount of the spent medium that is transferred to the culture during inoculation and also reduces the cost of production.

The widely used low MOI strategy for producing recombinant proteins and products such as virus-like particles, results in an unsynchronized infection so that the population lacks a uniformity of metabolic status.[169] As a consequence, the production of the defective interfering particles is increased and the target protein is exposed to proteases for long periods.[174] In addition, the time to harvest is extended and there is no clearly identifiable harvest time.[108]

The use of a high MOI strategy allows a more rapid infection, but requires a high titer of baculovirus for infection. Presence of defective interfering particles in the viral inoculum results in a reduced efficiency of viral expression and a decreased process performance. The cost of production is also increased.

An optimal MOI strategy depends on other factors such as the baculovirus, the cell line, the culture medium, the mode of operation of the bioreactor and the physiological state of the cells at infection.[27,175,176] The latter is related to the time of infection (TOI), i.e. the time elapsed since the initiation of a batch culture and the instance of infection. The TOI establishes the cell concentration at infection, i.e. the concentration of the cells just before the viral inoculum is added. The MOI is intrinsically related to the TOI as the concentration of cells determines the suitable value of MOI.

The effects of cell concentration at TOI on the production of baculovirus have been discussed in the literature.[176] Infection at low MOI progressively reduced the titer of baculovirus as the TOI was increased. Conversely, infection at a high MOI increased the viral titer as the TOI was increased. In view of these results, Carinhas et al.[176] recommended a combination of a low cell concentration at TOI and a low MOI as being best for propagation of the virus.

Downstream Processing

In production of baculoviruses for use as agricultural pesticides, the virus recovered from the clarified culture broth is generally used directly without purification.[29] In contrast, in production of most recombinant proteins and viruses for therapy[18] and vaccine delivery,[5,15,16] the product requires extensive purification downstream of the production culture. Therefore, there is an increasing need for highly efficient and robust downstream processes for baculovirus-based products.[177]

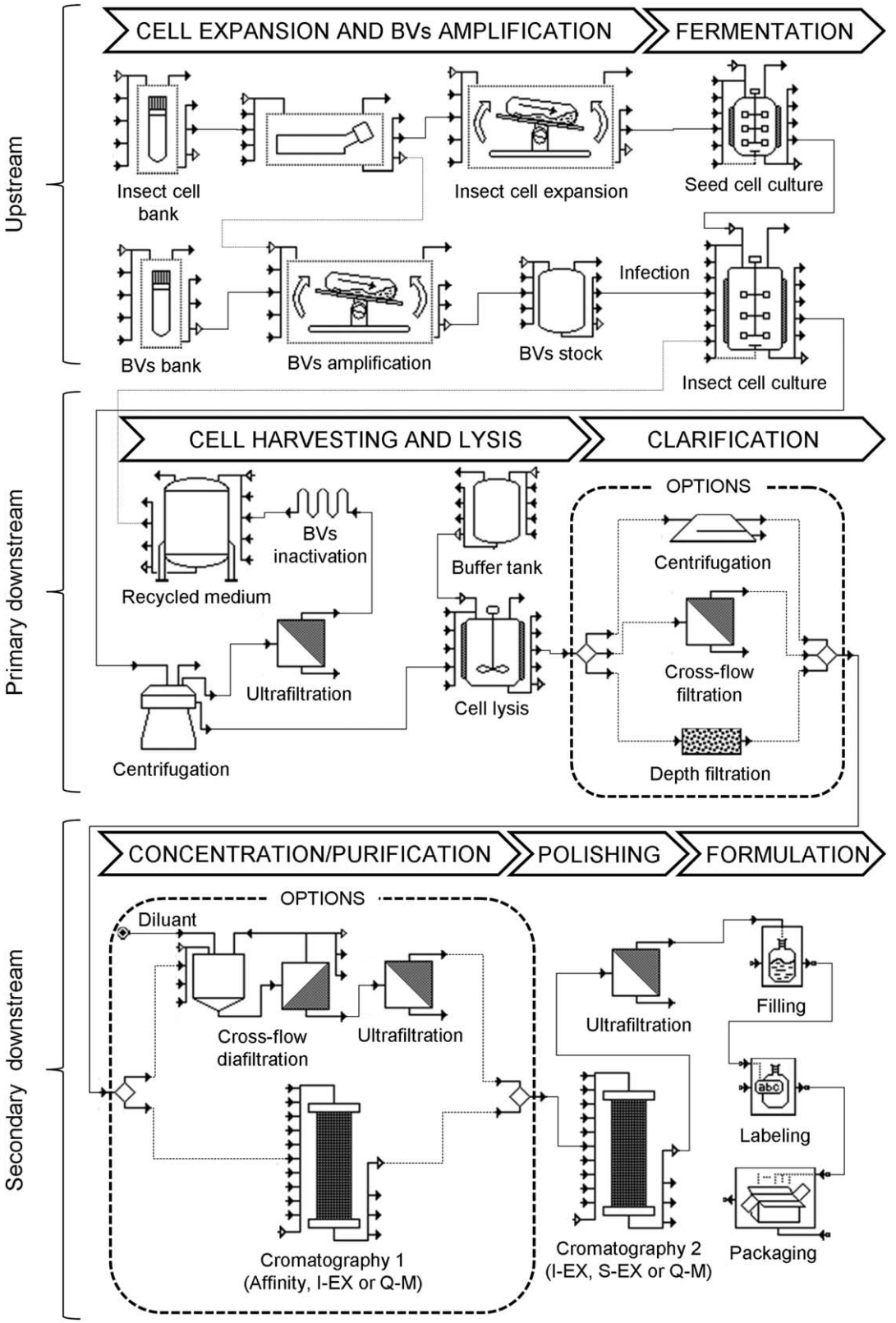

Figure 4. A conventional BEVS-IC-based scheme for producing recombinant proteins. The processing units grouped within a dashed box represent the different options available for a given operation. Commonly several operations are combined in series. An optional scheme involving recycle of the spent medium is also shown. I-EX: ion exchange; Q-M: Q-membrane; S-EX: size exclusion.

The specifics of a product recovery and purification process depend on the type of the product (e.g. viral particles, proteins) and its characteristics such as the structure, molecular weight, the nature of glycosylation and whether the product is intracellular or extracellular.[177,178] In addition, the downstream process is influenced by the nature of the impurities and whether the medium contained serum, or was serum-free.[177,178] The mode of operation of the culture and

Table 3. Analytical Methods used in Assuring Product Quality and Consistency (Based on Vicente et al.[15])

Attribute	Analyte	Assay
Quantity	Viral proteins	Total proteins; ELISA; surface plasmon resonance
Identity	Viral proteins	ELISA; restriction enzyme analysis; oligosaccharide mapping; southern blot analysis; western blot analysis; SDS-PAGE
Purity	Viral proteins. Host cell proteins. Residual host cell DNA. Residual baculovirus	Protein sequencing. Total proteins; ELISA. Real-time qPCR. Immunoblotting; DNA staining
Quality (activity and potency)	Viral proteins	In vitro (cell-based potency assay) and in vivo tests

the scale of operation also influence the downstream process.[177] A downstream recovery process should provide a product with the desired concentration, purity and the other quality attributes at a minimal cost.[178]

Ultracentrifugation has been commonly used to concentrate and purify baculovirus vectors,[179] but this method is inefficient. It has a poor reproducibility and limited scalability.[180] Up to 50% of the initial viral titer is lost as a result of particle aggregation.[180] A combination of ultracentrifugation and size exclusion chromatography has been claimed to reduce the loss of yield by up to 25%.[177,181]

More refined virus recovery strategies include the use of cation-exchange chromatography,[182] diafiltration using a cross-flow ultrafiltration module[183] and concentration of biotinylated baculovirus vectors by magnetic beads.[184] All these methods have a limited scalability,[177] but may be quite satisfactory as the scale of BEVS production operations is generally smaller than 5,000 L. An efficient downstream processing strategy based on disposable technology consisting of depth filtration, ultrafiltration/diafiltration and anion exchange membrane adsorption has been reported.[185,186] Membrane filtration processes are further discussed in the literature.[132]

Downstream operations for the recovery of BEVS recombinant proteins typically require an initial clarification step followed by concentration/purification steps and subsequent polishing of the product.[15,177] The clarification step removes large aggregates, empty capsids and cell debris from the broth. In some cases protease inhibitors may be added to suppress proteolysis of the product.[178] Clarification is generally achieved by centrifugation of the broth, but cross-flow microfiltration and depth filtration processes[132] may be used effectively with recovery yields commonly exceeding 90%.[187]

The concentration and purification steps following clarification are designed to remove proteases, endotoxins, pyrogens, and viral and cellular DNA, while reducing the total volume. An initial cross-flow diafiltration and subsequent concentration by cross-flow ultrafiltration are the processes of choice.[15] Other commonly used purification/concentration methods involve chromatographic processes based on affinity ligands,[188] ion-exchange,[189,190] and membranes.[191]

The polishing steps reduce the residual trace impurities (mainly DNA and host cell proteins) to acceptable levels. Polishing generally relies on chromatographic methods. Ion-exchange chromatography[192] and size-exclusion chromatography[193] are commonly used. Increasingly, single use disposable downstream processing equipment, is becoming the norm in newer downstream process designs[185,186,194] as this reduces the potential for contamination of the product.

A general process for producing BEVS-IC derived proteins is shown in Figure 4. The process consists of steps for cell expansion, virus amplification, insect cell culture, infection, harvest and further downstream purification operations. Multiple options available to achieve certain objectives of downstream processing and product purification are shown.

Quality Control of Proteins Produced Using the BEVS-IC System

The design/implementation of a large-scale protein production/purification process using the BEVS-IC system depend on the variables discussed earlier in this review, including: the baculovirus; the host cell; the composition of the cell culture medium; the culture methodology and environmental conditions; the specifics of the product; and the nature of the impurities present. A product may be potentially contaminated with materials derived from the virus, the cell and the culture medium. Attaining the specified level of purity is especially important for products that are intended for parenteral use and for in vitro diagnostics in which a contaminant could lead to a false result. The acceptable levels of impurities may be specified by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) in consultation with the manufacturer. In addition to meeting these quality standards, the EMA and the FDA require the production process to be of an approved design. The process must be operated in accordance with the standard operating procedures that have been validated to provide a product of the desired quality.[195]

The use of validated analytical methods performed by appropriately trained personnel, is essential to assuring the product quality. Although the BEVS-IC system is widely used for producing recombinant proteins, there is a lack of standardized quality assurance methods. Each new production process and its product are assessed on their own merit for obtaining marketing approval from the regulatory bodies. In attempts to simplify the effort required for gaining approvals, the International Society for Bioprocess Technology has hosted initiatives to develop protocols for characterizing BEVS-IC products.[196]

The characterization of quantity, identity, purity and quality of BEVS-IC produced proteins requires the use of specifically designed analytical methods.[177] The typically used methods are summarized in Table 3. The quantification and characterization of recombinant proteins have traditionally used methods such as the total protein assay, ELISA, western blot, SDS-PAGE, qPCR, and electron microscopy.[177] These methods are certainly useful, but they are time consuming, laborious, and of low sensitivity. More rapid and sensitive analytical methods applicable over the entire production chain are desired. Some of the new promising methods are monolithic HPLC and two-dimensional fluorometry. Monolithic HPLC columns can be used to monitor the product of interest through various stages of the production process.[197] Two-dimensional fluorometry is a noninvasive technique that uses

Table 4. Relevant Vaccines Candidates for Human and Animal Use in Clinical and Preclinical Development Produced Using BEVS-IC (Based on Cox[12])

Disease	Protective Antigen	Clinical Status	Reference
Diabetes	GAD	Phase III	201
Hepatitis E	ORF 2	Phase II*	202
Influenza	NA	Phase II	203
Influenza	HA, NA, M1	Phase II	204
ParvovirusB-19	Parvovirus VLP	Phase II	205
Influenza H5	HA	Phase I	206
Norwalk	Capsid VLP	Phase I	207
Respiratory syncytial virus	F protein VLP	Phase I	208
Rotavirus	VP2, VP6, VP7 VLP	Preclinical	209
Ebola	VP40 VLP	Preclinical	210
HIV	Pr55gag, envelope VLP	Preclinical	211
Chikungunya virus	C, E3, E2, 6K, E1, VLP	Preclinical	212
Feline calicivirus	VP1 VLP	Veterinary (animal studies)	213
Papillomavirus	L1, L2 VLP	Veterinary (animal studies)	214
Goose Parvovirus	VP1, VP2, VP3 VLP	Veterinary (animal studies)	215
Porcine encephalomyocarditis	P1, 2A, 3C VLP	Veterinary (animal studies)	216
Foot and mouth disease	P1, 2A, 3C VLP	Veterinary (animal studies)	217
Rotavirus	VP6, VP7, VP8 VLP	Veterinary (animal studies)	218

*Phase II trials revealed the vaccine to be more than 95% effective in preventing infection, but GSK did not take the vaccine forward to production.

optical fibers to detect the fluorescence emitted by certain compounds (e.g. proteins) both inside and outside the cells, to potentially allow online control of proteins expression.[198] Quality characterization of parenteral recombinant proteins is further discussed in the literature.[177,199,200]

Proteins Produced Using the BEVS-IC System

So far, seven protein-based products made using the BEVS-IC appear to have been commercialized. Four of these are vaccines for veterinary use and the remaining three are products for human parenteral use. The veterinary vaccines Porcilis Pesti™ (Merck; www.merck.com) and Bayovac™ (Bayer; www.bayer.com) protect against the classical swine fever virus. Porcilis Pesti™ (Merck) became commercially available in 2000. The vaccines CircoFLEX™ (Boehringer Ingelheim; www.boehringer-ingelheim.com) and Porcilis™ PCV are for protection against the porcine circovirus type 2.[6,28]

The first BEVS-IC derived product for human use was licensed by EMA in 2007 and by FDA in 2009. This is the now widely used vaccine Cervarix™ produced by GlaxoSmithKline (www.gsk.com) to protect against cervical cancer caused by a human papilloma virus.[6] The other BEVS-IC derived products licenced for human use are Provenge™ produced by Dendreon (www.dendreon.com) and FluBlok™ produced by Protein Sciences Corporation (www.proteinsciences.com). Provenge™ was licensed by the FDA in 2010 for the treatment of prostate cancer.[6] FluBlok™ has been licensed by the FDA in 2013 for active immunization against disease caused by influenza virus subtypes A and type B.[9,28]

The next vaccine product to be licenced would likely be Diamyd™, a therapeutic vaccine for diabetes type 1 being developed by Diamyd Medical (www.diamyd.com). This product is in phase III clinical trials. Several other protein-based and VLP-based vaccine candidates for influenza, HIV, rotavirus, norovirus, hepatitis, malaria, and other diseases are undergoing clinical trials or preclinical evaluations. Yet other products are being assessed for veterinary use. These products have been the subject of recent reviews.[6,9,17,28] Some of the vaccines in development are shown Table 4.

From a production perspective, the manufacture of vaccines poses particular challenges. The quantities required may be relatively small and the cost generally must remain low. A manufacturer may need to rapidly respond to emerging mutants of an existing pathogen, or may be faced with a sudden outbreak of a highly infectious disease.[219] Often, a new vaccine must be developed and put into commercial production at short notice. A BEVS-IC based production of some vaccines may have to be designed for responding flexibly to changing demand scenarios. The BEVS-IC based production of vaccines has been claimed to be relatively quick and straightforward to implement,[6,9] for example, in response to a developing pandemic.[6,9] The time from receipt of an influenza virus to preparation of a master cell bank of the recombinant baculovirus harboring the vaccine antigen can be as short as 30 days. Therefore, the production of a suitable commercial vaccine can commence within about 45 days from receipt of the etiologic agent.[9]

The development and production of vaccines can be expensive. For example, the FluBlok™ vaccine cost an estimated $100 million to become licenced.[6] Consequently, the progress in developing recombinant vaccines is most rapid generally only for products that can be sold for a high price (e.g. the vaccine for human papilloma virus), or for those being developed through public financing.[6]

Much of the cost is associated with the development and licencing of a product, not with its actual manufacture. Notwithstanding this, the cost of production can be reduced substantially by improving productivity. This needs to happen at the earliest stages of product development and process design. For example, through the use of alternative baculovirus promoters, a judicious selection of the baculovirus and the host cell, design of a suitable cell culture medium, selection of an appropriate bioreactor and its mode of operation, and the development of a robust, scalable and efficient downstream process.

Concluding Remarks

BEVS is an important and powerful method for producing recombinant proteins. Progress is being made in genetic manipulation of the viral vectors to improve protein expression and quality. In addition, the insect cell lines are being engineered for improved capabilities in protein folding and

glycosylation. The use of improved culture media, bioreactor designs, and operational strategies in combination with investments in process optimization are enhancing the capabilities of the BEVSs. Downstream product recovery and purification methods are generally well established certainly at small and medium scales of operation. Increasing use of disposable process equipment is enhancing the safety of the products and reducing losses associated with contamination. In view of the continuing advances in all the relevant areas, the use of BEVSs will further expand.

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