CHAPTER THREE

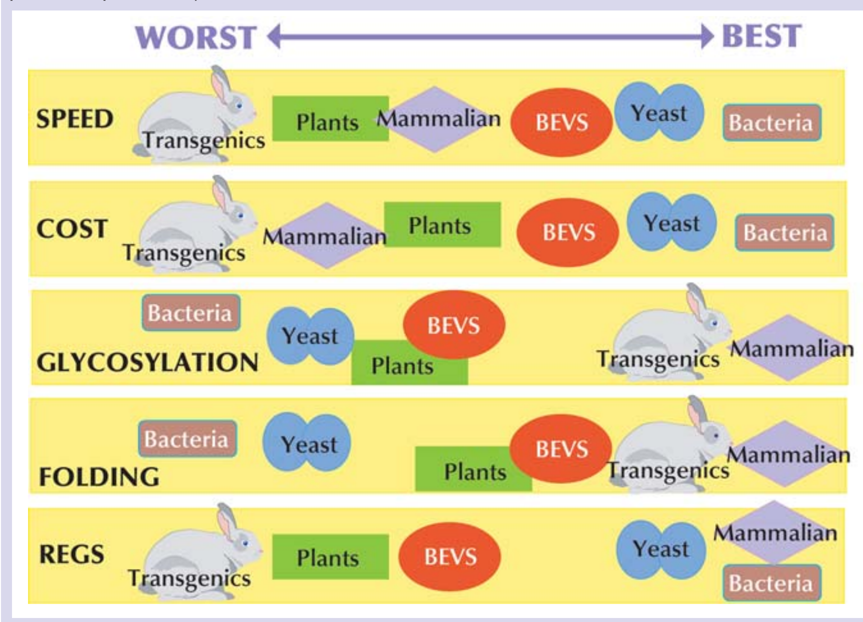
Commercial Production in Insect Cells

One Company's Perspective

by Manon M.J. Cox

The human genome project sequenced more than 30,000 genes, all of them potential disease targets. Identifying the function of those genes, making the proteins they encoded, characterizing those proteins, and finding applications for them is generally considered the biotechnology industry's next challenge. Factors such as time to market, cost of goods, product characteristics, regulatory hurdles, and intellectual property can ultimately determine the success or failure of a given project. Selecting an appropriate expression system for the protein of interest will affect most of those factors. For any protein of interest, the critical parameters must be identified before the most optimal expression system can be found. Figure 1 provides some basic guidance for selecting an expression system.

Figure 1: The author's qualitative ranking showing strengths and weaknesses of various protein expression systems.



The baculovirus expression vector system (BEVS) is best known and used as a research tool. Thousands of articles describing the use of the BEVS system for the production of proteins for research use have been published during the past few decades. Its reputation is one of providing quick access to biologically active proteins. But today no protein produced in insect cells has been approved for human therapeutic or vaccine use. The case study presented in this chapter provides evidence that this technology continues to have tremendous potential.

Science

Baculoviruses are commonly found on green vegetables, and are therefore part of the diet of healthy individuals. The name is derived from the Latin *baculum* (rod), which describes the shape of the virus. Baculoviruses are characterized by a narrow host range (1), mainly lepidopteran insect species (moths and butterflies), in which they cause fatal disease. Within days, a baculovirus-infected insect will transform into a glue-like structure mostly consisting of polyhedrin protein. In the virus's natural habitat, polyhedrin protects it from ultraviolet light. Scientists demonstrated in the 1980s that the DNA encoding the protein was unnecessary for the survival of the virus in a laboratory, so it could be replaced by genes encoding proteins of medical importance, such as interferon (2). Insect cells can perform many of the posttranslational modifications required for the biological activity of many complex proteins: e.g., glycosylation, disulfide bond

formation, and phosphorylation (**3**). Over the years, many thousands of genes have been successfully cloned and expressed in insect cells.

With the BEVS system, proteins can be produced in weeks rather than in months or years because the virus (rather than the cell line itself) is genetically modified to infect insect cells.

The baculovirus genome is relatively small (about 160 kb), and it can be easily characterized using genome digesting and Southern blotting techniques. After the virus infects an insect cell, the cell is transformed into a baculovirus protein production facility before it dies. Heterologous proteins that would be lethal to the cells could be produced during the short remainder of their lifespan. The protein of interest is usually made under control of the polyhedrin promoter, which is one of the strongest known promoters. Insect-cell–produced proteins are generally bioactive because of the cells' ability to perform complicated posttranslational modifications.

To date no approved BEVS-made product is on the market for human vaccine or therapeutic use, even though a number of products are in advanced clinical trials. For example, Dendreon Corporation (www.dendreon.com) is conducting phase III clinical studies of its prostate cancer vaccine candidate produced by insect cells. GlaxoSmithKline (www.gsk.com) is performing clinical studies with a human papilloma virus vaccine candidate, and Protein Sciences Corporation plans to initiate a phase III trial for its influenza vaccine candidate in the fall of 2004.

A major challenge is presented by the fairly complex patent landscape surrounding the expression system. Over 5000 patents refer to the use of insect cells or baculovirus, varying among multiple cell lines, signal sequences, promoters, dual-promoter systems, and the production of specific types of proteins. It is therefore recommended to conduct a directed patent study toward any application envisioned before embarking on the path of this expression system.

An absence of complex sugars in BEVS-produced proteins raises questions relating to their biological activity. But the presence of relatively simple glycosylation can be an advantage for some proteins and a disadvantage for others.

Protein Sciences Corporation (PSC) has developed technology for large-scale (600 L) production of proteins in insect cells using the BEVS. The company has tested recombinant protein vaccines for AIDS, malaria, cancer, and for viral influenza in phase I and II human clinical trials, establishing a regulatory track record.

Technology Development

PSC developed immortal insect cell lines from the ovaries of *Spodoptera frugiperda,* referred to as Sf21 and Sf9 cells, and a serum-free cell culture line with defined growth characteristics (**4**). The serum-free cell line was selected to grow at high densities in suspension without clumping. Cell doubling time varies from 18 to 24 hours, depending on the selected cultivation system (spinner and shaker flasks, bioreactors of varying size). Cells can be passaged more than 50 times while maintaining a high viability. They are also highly susceptible to baculovirus infection, typically resulting in high-titer virus stock around 10^8 plaque forming units (pfu) per mL and accordingly high protein production levels. Figure 2 shows the various stages of infection in the serum-free cell line. The cells increase about fourfold in size during infection, increasing biomass and protein production.

Signal Sequence: Many heterologous proteins are secreted into the growth medium. We identified a baculovirus signal sequence (derived from a 61-kDa membrane-associated glycoprotein), which increased secretion and expression of heterologous proteins (**5, 6**). The SDS-PAGE gel in Figure 3 compares vascular endothelial growth factor (VEGF) expressed in insect cells using its natural signal sequence with the 61-kDa–derived signal sequence. Analyzing the gel by densitometry suggested that expression levels obtained with the baculovirus-derived signal sequence are about tenfold higher than with the natural signal sequence. The right panel shows an immunoblot demonstrating the correct identity of the protein.

Protease-Deficient Baculovirus Vector: Some heterologous proteins are prone to protease degradation.

Figure 2: Infection of SF+ cells at MOI = 0.2 (as measured in a standard 1-hour plaque assay). The cells can be synchronously infected, and by 27 hours postinfection, all show typical morphological changes. Late in infection (75 hours), the cells have enlarged to more than twice normal size, but the majority of them remain intact.

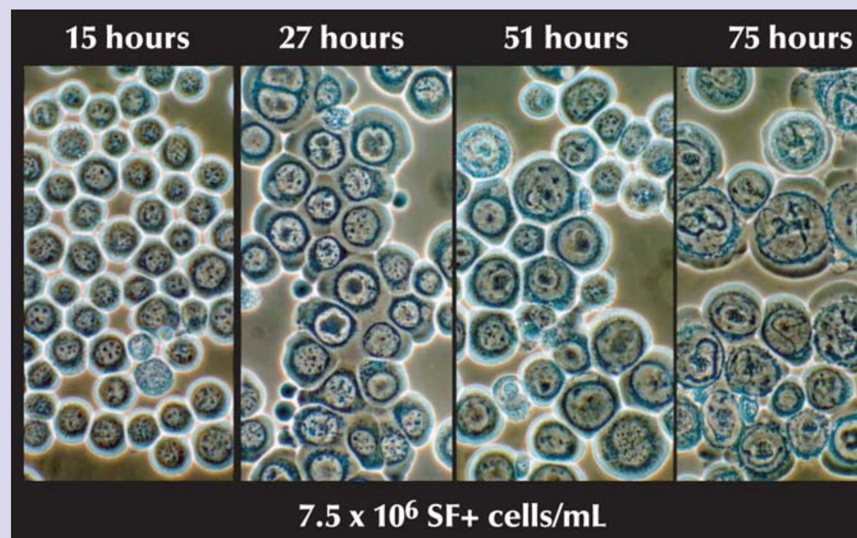
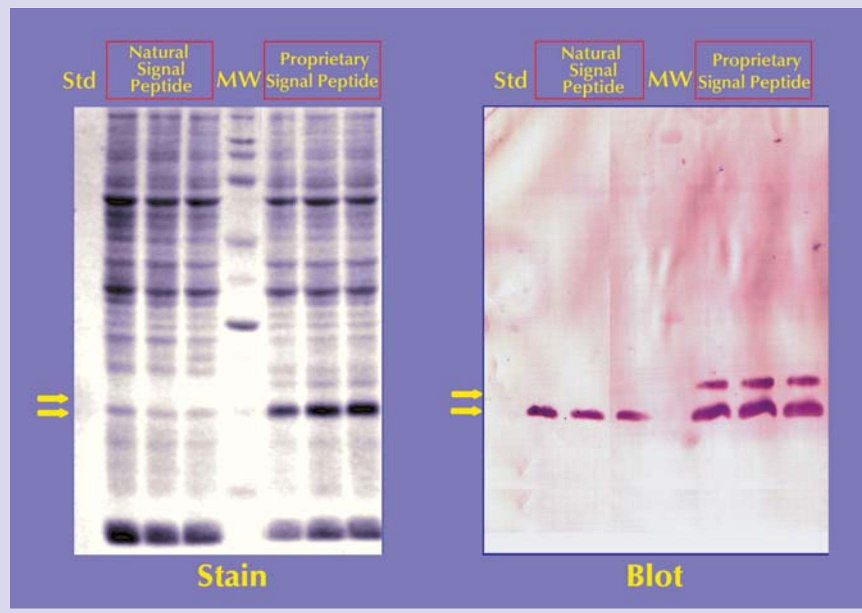


Figure 3: Expression of VEGF showing advantages of the PSC signal sequence. VEGF was expressed either with its natural signal sequence or with the 61-kDa signal sequence. Left panel shows a Coomassie-stained SDS-PAGE gel; right panel shows a Western blot using VEGF-specific antibody for confirmation of identity.



Early during the BEVS infection process, more protein may be degraded than is being produced. A pragmatic approach to overcome this degradation would be to harvest the protein of interest early in the infection process — or to add protease inhibitors. PSC created a baculovirus backbone that lacks the cathepsin gene and has an incomplete chitinase gene. Figure 4 illustrates its use for the production of certain proteins. However, it does not result in better production levels in all cases.

High Cell Density: Insect cells are susceptible to infection while they are in their logarithmic growth phase, which limits the potential for generating high concentrations of biomass before producing the protein of interest. Under normal cultivation conditions, insect cells can be successfully infected at a density of 2–3 million cells/mL. Our fermentation conditions use a dialysis fermentation set-up under which cells can be infected at higher cell densities (15–20 million cells/mL).

The dialysis set-up is based on waste products being removed from the media while fresh ingredients are added without the cumbersome process of identifying critical components. It provides favorable oxygen, nutrient supplies, and reduced shear forces necessary for high-density cell propagation. These include continuous circulation of cells from the bioreactor, through a semipermeable hollow-fiber filter, then back to the bioreactor in a manner analogous to the circulation of blood through the kidneys (with inline oxygenation as in the lungs).

Upstream Processing: The clarification step needs to remove DNA, lipids, and other contaminating agents from the cell culture medium. Addition of calcium chloride to a concentration of 10 mM and Tris base to a concentration of 28 mM at pH 8.0 allows the formation of a calcium gel that binds DNA and can be separated easily from the supernatant by centrifugation. A drawback to this method is that certain calcium-dependent proteases may be activated during the process, which can lead to degradation of the protein of interest and a reduction of the overall process yield. Adding chelating agents after centrifugation may reduce this effect.

Large-Scale Production Capacity: Producing proteins in insect cells at larger scales requires a high-titer working virus bank and an efficient insect cell infection process. The high–cell-density technology might offer a future solution, but it is not yet used in our production facility. The serum-free insect cell line produces high-titer virus stocks, thereby reducing the amount of virus that needs to be added to the bioreactor to about 1% of the total reactor volume. Currently the process is performed at a 600-L scale, and no major hurdles are envisioned when scaling the process 10 to 50-fold.

Clinical and Regulatory History

We have tested a number of recombinant proteins made in insect cells as vaccine candidates for the prevention of various diseases, including AIDS, malaria, colon cancer, and influenza. To date more than 50,000 doses of protein have been administered in more than 5000 subjects and patients without reportable adverse effects. The data suggest that BEVS is a safe manufacturing system for protein production. Our drug master file contains general cell line, process, and company information derived from six previously filed INDs that can be cross referenced by its collaborators.

One Application

In 1997, health officials in Hong Kong were alarmed by the death of a child following infection with a highly pathogenic avian H5N1 influenza strain. The virus had previously caused the death of 70–100% of the chickens in infected flocks there. Before year's end, six of 18 infected people died of the disease (**7**). Fortunately, the efficiency of virus transmission between humans was low, but the need for better vaccines became obvious (**8**). Particularly alarming was that the usual egg-based influenza vaccine manufacturing process cannot produce a vaccine for this kind of virus because the chicken embryos used for vaccine production are killed by this highly pathogenic virus.

The insect-cell–based production technology offered a solution. The cDNA encoding the hemagglutinin (HA) gene from the avian H5N1

strain was used to produce a subunit vaccine. Within six weeks, PSC made HA antigen for testing in chickens. Tests confirmed immunogenicity — and more important, protection from a lethal viral challenge — in chickens (**9**). Four weeks thereafter, 1700 doses were delivered to NIH for testing in humans. The company received FDA approval for compassionate use. A clinical study was conducted using the material produced. And the studies suggested that the BEVS-expressed H5 HA induced functional antibodies in individuals who had no prior exposure to the H5 viruses (**10**).

The BEVS technology can be a powerful manufacturing technology to provide health care solutions to pandemic, biodefense, and emergency scenarios. We are exploring the potential of this production technology for rapid development of a SARS subunit vaccine. A recombinant trivalent hemagglutinin influenza vaccine is being tested in a large US phase II(b) clinical study in the elderly. For those exploring the BEVS technology as a commercial large scale production system, the company offers process development services.

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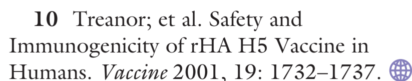

***Manon M.J. Cox** is chief operating officer at Protein Sciences Corporation, Meriden, CT 06450. Previously she was with Gist-brocades (now part of DSM), a Dutch company specializing in fermentation, where she held various management positions, most recently in new business development, and before that in research and development (R&D) and production. Before joining Gist-brocades, she was a molecular biologist on the development of a PCR screening test for cervical cancer at the University of Amsterdam. She received her MBA with distinction from the University of Nijenrode and the University of Rochester, NY, and a PhD in molecular biology from the University of Nijmegen, The Netherlands.*

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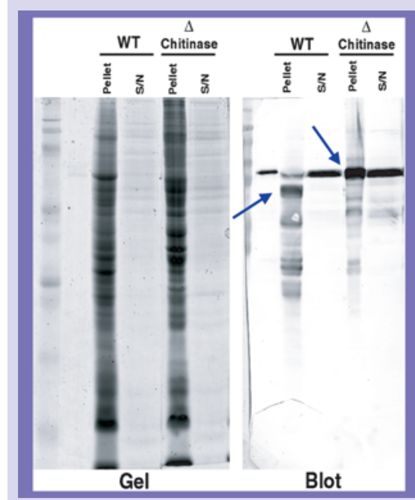
Figure 4: Protease-deficient baculovirus. Protein X was cloned in a baculovirus backbone that lacks the cathepsin gene and part of the chitinase gene found in the wild type baculovirus. As shown in this Western blot, more intracellular full-length protein can be detected when using the protease-deficient backbone.



For Further Reading

Ikonomou, L; Schneider YJ; Agathos SN. Insect Cell Culture for Industrial Production of Recombinant Proteins. *Appl. Microbiol. Biotechnol.* 2003, 62(1): 1–20.

From the Abstract: Our current knowledge of insect cell metabolism is summarized, and emphasis is placed on elements useful in the rational design of serum-free media. The culture of insect cells in the absence of serum is reaching maturity, and promising serum substitutes (hydrolysates, new growth and production-enhancing factors) are being evaluated. Proteolysis is a problem of the BEVS system due to its lytic nature, and can, therefore, be a critical issue in insect cell bioprocessing. Several cell- or baculovirus proteases are involved in degradation events during protein production by insect cells. Methods for control, the optimal inhibitors and culture and storage conditions which affect proteolysis are discussed. Finally, engineering issues related to high-density culture (new bioreactor types, gas exchange, feeding strategies) are addressed in view of their relevance to large-scale culture.

See also:

Griffiths CM; Page MJ. Production of Heterologous Proteins Using the Baculovirus/Insect Expression System. *Methods Mol. Biol.* 1997, 75: 427–440.

Wang, M; Cox, M. Baculovirus Protein Expression Vector System Tutorial: Optimized Sf+ Cell Line, Meda, Baculovirus-Derived Signal Peptide, and Promoter Efficiently Provide High Yields. *Gen. Eng. News* 23(20), 15 November 2003.

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