# Chapter 8
# Insect Biotechnology

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**Abstract** For the purpose of this work, insect biotechnology, which is also known as yellow biotechnology, is the use of insects as well as insect-derived cells or molecules in medical (red biotechnology), agricultural (green biotechnology), and industrial (white) biotechnology. It is based on the application of biotechnological techniques on insects or their cells to develop products or services for human use. Such products are then applied in agriculture, medicine, and industrial biotechnology. Insect biotechnology has proven to be a useful resource in diverse industries, especially for the production of industrial enzymes including chitinases and cellulases, pharmaceuticals, microbial insecticides, insect genes, and many other substances. Insect cells (ICs), and particularly lepidopteran cells, constitute a competitive strategy to mammalian cells for the manufacturing of biotechnology products. Among the wide range of methods and expression hosts available for the production of biotech products, ICs are ideal for the production of complex proteins requiring extensive posttranslational modification. The progress so far made in insect biotechnology essentially derives from scientific breakthroughs in molecular biology, especially with the advances in techniques that allow genetic manipulation of organisms and cells. Insect biotechnology has grown tremendously in the last 30 years.

## Abbreviations

| | |
|---|---|
| AAV | Adeno-associated virus |
| AcMNPV | *Autographa californica* multinuclear polyhedrosis virus |
| Bt | *Bacillus thuringiensis* |
| BV | Baculovirus |
| Cry | Crystal protein |
| EGFP | Enhanced green fluorescent protein |
| FXR | Farnesoid X-activated receptor |
| GFP | Green fluorescent protein |

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| | |
|---|---|
| GM | Genetically modified |
| HR | Herbicide resistant |
| BEV | Baculovirus expression vector |
| IC-BEVS | Insect cell baculovirus expression vector system |
| IC | Insect cell |
| IR | Insect resistant |
| LXR | Liver X receptor |
| LV | Lentivirus |
| MOI | Multiplicity of infection |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| NPV | Nucleopolyhedrosis virus |
| rAAV | Recombinant adeno-associated virus |
| rTRAIL | Recombinant TNF-related apoptosis-inducing ligand |
| rBV | Recombinant baculovirus |
| VEGF | Vascular endothelial growth factor |
| VLP | Viruslike particle |

## 8.1 Introduction and Scope

For the purpose of this work, insect biotechnology, which is also known as yellow biotechnology [1–3] in contrast to green (plant), red (animal), and white (industrial) biotechnology, is discussed as the application of biotechnological techniques on insects or their cells to develop products or services for human use. Such products are then applied in agriculture, medicine, and industrial biotechnology [3–6]. Insect biotechnology has proven to be a useful resource in diverse industries, especially for the production of industrial enzymes, microbial insecticides, insect genes, and many other substances. Insect cells (ICs), and particularly lepidopteran cells, constitute a competitive strategy to mammalian cells for the manufacturing of biotechnology products. Among the wide range of methods and expression hosts available for the production of biotech products, ICs are ideal for the production of complex proteins requiring extensive posttranslational modification.

The development of the field was driven by the importance of insects in all fields of human endeavor. Insects are the most diverse of all groups of organisms, with more species than any other. The number of extant species is estimated at about $1.9 \times 10^6$ and potentially represents over 90 % of the various animal life forms on Earth [7]. They have overwhelming impact on agriculture and public health. Insect biotechnology uses biotechnological methods on insects (or their cells) to develop products or services. These new substances have applications in medicine, the sustainable protection of plants, and industrial biotechnology. This means that insect biotechnology employs the general principles of biotechnology. More specifically, insect biotechnology borrows from and is driven by scientific breakthroughs in molecular biology, particularly by the development of tools and techniques that allow genetic characterization and engineering of organisms and cells as with

recombinant DNA technology and plant protoplast fusion. Recently, bacteria have been engineered to carry and express silk genes from the mulberry silkworm, *Bombyx mori*, and used in the production of synthetic silk, the natural protein fiber, some forms of which can be woven into textiles [8] (see also Chap. 10). As a basic research tool, the strategies of insect biotechnology include the sequencing and annotation of insect genomes as well as analyses using comparative genomics. Insect biotechnological applications to pest management include the development of resistant crops and trees that express insect-specific toxins, the design of microbial agents with enhanced insecticidal potency, and the engineering of insects that can transfer lethal genes to natural populations following their mass release in the field. Comparative genomics analyses also make it possible to identify insect-specific genes that can be targeted for rational insecticide design.

## 8.2 Applications of Insect Biotechnology in Agriculture

It has been shown by the USDA [9] and numerous other sources that insects and nematodes are the world's most important pests of agricultural plants and livestock. They have been shown to cause billions of dollars of losses to growers and livestock producers every year because of lowered yields, deterioration of quality, and disease. As already indicated earlier in this review, chemical pesticides are a major tool for insect and nematode control. However, safer alternatives to chemical pest control have become imperatives because most of these pesticides are potentially harmful to the ecosystem and human health. The advent of biotechnology in general and insect biotechnology in particular is a welcome development in the fight against insect pests.

Modern agricultural biotechnology or genetic engineering includes manipulation of the genetic makeup of organisms for use in the production or processing of agricultural products. A major strategy in increasing crop yield is the development of pest management regimens consisting of herbicide-resistant (HR) and insect-resistant (IR) crops as well as transgenic insects. Genetically modified (GM) crops first became commercially available in 1996. By 2008, 90 % of soybean, 78 % of cotton, 72 % of canola, and 60 % of maize hectares were cultivated globally with these GM crops [10]. The most well-known example of this genetic manipulation in both plants and viruses is the insertion into a plant or virus of the gene coding for the production of the delta endotoxin of *Bacillus thuringiensis* [11]. *B. thuringiensis* (or Bt) is a Gram-positive, soil-dwelling bacterium, commonly used as a biological pesticide. It also occurs naturally in the environment of mosquito larvae, moth, and butterfly caterpillars, as well as on leaf surfaces, aquatic environments, animal feces, insect-rich environments, flour mills, and granaries [12]. Many Bt strains produce "Cry" during sporulation. Cry (short for crystal proteins; Fig. 8.1), also called δ-endotoxins, are proteinaceous inclusions encoded by plasmid-borne *cry* genes and have insecticidal action on susceptible insects. Bt is remarkably nontoxic to humans, and to a large extent nontarget fauna, and is easy to use, making it a

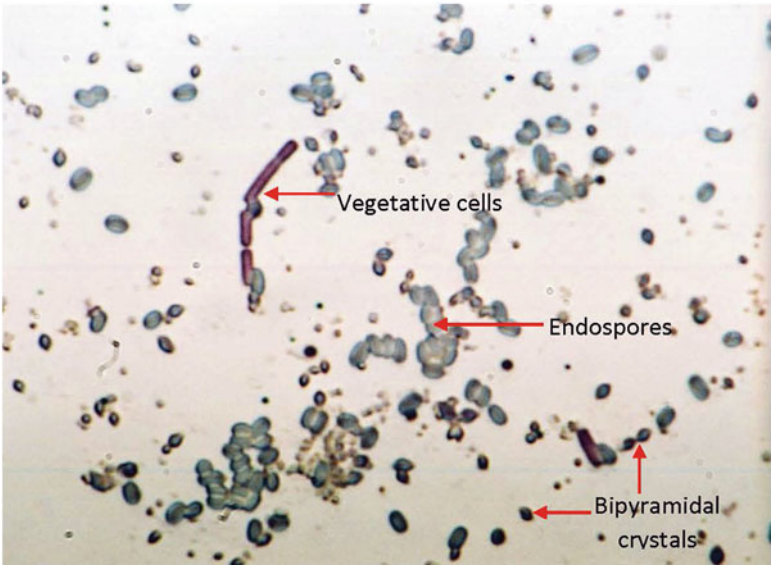


**Fig. 8.1** *Bacillus thuringiensis kurstaki* showing crystals, vegetative cells, and spores after staining by a differential crystal staining method. Isolated from soil in Tennessee

popular alternative to chemical treatments for crop protection. This has led to the use of Bt toxins as insecticides and, more recently, to GM crops expressing Bt proteins in transgenic plants.

### *8.2.1 About* Bacillus thuringiensis kurstaki

*B. thuringiensis* (Bt) *kurstaki* has served as a microbial insecticide for many decades, but the widespread use was limited by its instability when exposed to UV radiation under sunlight and its poor retention on plant surfaces in wet weather. The high toxicity of the Bt toxins to a variety of insect pests, and the ease with which the gene could be isolated from bacterial plasmids, made it an obvious choice for the development of the first insect-resistant transgenic plants. The active Bt toxin binds to a receptor in cells lining the insect midgut and creates a channel, allowing free passage of ions. The cells lining the midgut die, and very soon, the insect dies, too. Different strains of Bt contain plasmids encoding different toxins with different specificities of action against insects. A particular toxin is generally effective against only a limited range of closely related species. Bt toxins are used in a variety of transgenic crops, including cotton, for protection against a variety of lepidopteran pests; corn (maize), for protection against the European corn borer, *Ostrinia nubilalis* (Lepidoptera); and potatoes, for protection against the Colorado potato beetle, *Leptinotarsa decemlineata*. Intensive screening programs have led to important

collections of Bt strain isolates in the last few decades [13–18]. This became necessary as reports of the appearance of insect resistance and novel Cry proteins with toxic potential against different organisms emerged together with the need for more potent toxins with specificity for a much broader range of pests. The biosafety and mechanism of Bt toxin action are reviewed elsewhere [19–22].

## 8.3 Applications of Insect Biotechnology in Medicine

Reports abound on the emergence of new bacterial strains and increasing bacterial resistance against antibiotics leading to a growing number of severe, sometimes life-threatening bacterial infections in hospitals and even in daily life environment. A recent example is the 2011 *Escherichia coli* O104:H4 outbreak in Germany [44]. Reports of this nature clearly demonstrate the urgent need for new antimicrobial drugs. Insects have been shown to produce an array of antimicrobial metabolites which have potential as templates for further drug development.

In 1999, Bulet and colleagues reviewed the arsenal of agents used by insects to fight off invading microorganisms [45]. They reported that it is essentially based on short cationic antibacterial and antifungal peptides/polypeptides (see also Chap. 6). Depending on the species, insect adipose tissue can respond to bacterial infection by the synthesis of a large array of antimicrobial substances (e.g., *Drosophila* adults produce more than ten different molecules, including drosocin, drosomycin, defensin, metchnikowins, diptericin, and cecropins) or by producing relatively few molecules (e.g., a single peptide, defensin, in the dragonfly, *Aeshna cyanea*) [46]. Antimicrobial peptides produced by insects are structurally similar to those produced by many plants and animals. Industry has capitalized on that feature for the development of many new drugs from insects. For example, cecropins [47] have similarities with the amphibian α-helical antimicrobial peptides, magainins, found in frog skin secretions, while drosomycin from *Drosophila* and thanatin from the spined soldier bug, *Podisus maculiventris*, have similarities with plant defensins and frog skin secretion antimicrobial peptides, respectively. Bulet and colleagues [48] concluded that insect antimicrobial peptides are useful in overcoming the problem of acquired bacterial drug resistance and of the emergence of opportunistic pathogens, especially in immunosuppressed hosts due to the fact that:

1. They rapidly kill target microorganisms.
2. They have broad activity spectra.
3. They are not active against mammalian cells.
4. They have a mode of action that should restrict the selection of resistant strains.

A more recent review of insect products and their development as drugs published in 2014 revealed the importance of insect biotechnology in medicine. Based on knowledge gleaned from insect folk medicines, Ratcliffe and colleagues [4] described modern research into bioengineering of honey and venom from bees, silk, cantharidin, antimicrobial peptides, and maggot secretions and anticoagulants from bloodsucking insects into medicines. They described how modern molecular and

**Table 8.1** Examples of potential use of products of insect biotechnology in medicine

| Product | Source | Uses | Reference |
|---|---|---|---|
| 5.8 kDa honey component of manuka honey | *Apis* spp. | Stimulates TNF-α production via TLR4 in human monocyte cultures. For wound repair/healing | [23] |
| Manuka honey, buckwheat honey, honey products of Revamil, propolis | *Apis* spp. | Inhibits Gram-positive MRSA, vancomycin-sensitive and vancomycin-resistant *Enterococci, Streptococcus* species isolated from wounds, Gram-negative bacteria associated with wounds such as *Pseudomonas aeruginosa*, *Stenotrophomonas* species, and *Acinetobacter baumannii* | [24–29] |
| Quercetin, apigenin, and acacetin | *Apis* spp. | Anticancer, enhances the apoptotic ability of anti-CD95 and rTRAIL in acute lymphocytic leukemia | [30–32] |
| Bee, wasp, and ant venom products, e.g., melittin | *Asp* spp., *Vespula* spp., *Solenopsis* spp., *Pachycondyla* spp., and *Myrmecia* spp. | Inhibits or kills cancer cell types including hepatic, melanoma, ovarian, osteosarcoma, leukemic, prostate, mammary gland cells, bladder, and renal | [33–35] |
| Maggot alloferons | *Lucilia sericata* | Antiviral and antitumor activities | [36] |
| Cantharidin | Blister beetles, *Mylabris caragnae*, and *Mylabris phalerate* | Treatment of cancers including hepatic, colorectal, melanoma, pancreatic, bladder, breast, and leukemia as well as activities against *Plasmodium falciparum* and *Leishmania major* | [37–41] |
| Anopheline | *Anopheles* salivary gland | Thrombin inhibition and development of antithrombotics for use as anticoagulants | [42, 43] |

biochemical techniques have made it feasible to manipulate and bioengineer insect natural products into modern medicines. A summary of some of the most important compounds and their uses in medicine is presented in Table 8.1.

## 8.4 The Role of Insect Cell Cultures in Insect Biotechnology

Perhaps one of the areas in which insect biotechnology has made the greatest contribution to basic research is the development of insect cell cultures. In general, the establishment of cell cultures (animals, plants, insects, and fungi) is done by growing cells under conditions suitable for propagating stable, likely clonal, lines of cells harvested from embryos, specific tissues, or whole organisms in a variety of culture media developed for the species under investigation as described in Sect. 5. The justification for this is that it is easier to do experiments on individual cells than in animals, considering that the cell is the basic unit of life. It is also easier to produce large

quantities of metabolites, especially heterologous proteins, from cell cultures than from animals. ICs are also excellent systems for the production and posttranslational modification of large quantities of modified eukaryotic proteins in a short time.

The critical issues that must be considered for the successful establishment and maintenance of insect cell cultures [49, 50] are as follows:

1. The use of actively growing cells (i.e., cells in exponential growth phase) to start the culture. Cells such as the Sf9 and Sf21 may be obtained from the American Type Culture Collection. A temperature of 27–28 °C is optimal for growing these cells. Starter media such as TC-100 medium (Sigma, Cat# T3160) supplemented with heat inactivated 10 % fetal bovine serum (FBS) double in 20–24 h and do not require $CO_2$. Mammalian sera contain complement which mediates quick inactivation of baculoviruses (BVs). It is important to start cells from a frozen cell stock to avoid contamination. High-quality insect cells are crucial for the success of BV expression. Clumping could be a problem and should be avoided. Avoiding media particles could help reduce clumping, and gentle shaking in shake flasks could help dislodge them.
2. The importance of medium quality cannot be overemphasized. Synthetic serum-free media are available from Invitrogen and other suppliers and are hassle-free. Certain media can be preferred for certain applications. For example, synthetic serum-free media are strongly recommended for the expression of secreted proteins but can be also used for non-secreted protein studies and for transfections.
3. Transition from serum-containing to synthetic serum-free medium (weaning) is done gradually on healthy cells growing in log phase. It is done by slowly decreasing the percentage of serum-containing medium and increasing the percentage of synthetic serum-free medium in the cell culture over a period of about a week.
4. Antibiotics may be used to control contamination. An example is penicillin/streptomycin solution (Invitrogen SKU# 15070–063) used at 1:99 dilution. Gentamycin and nystatin can also be used routinely in insect cell culture. The use of antibiotics is optional.
5. Insect cells such as Sf9 or Sf21 cells can be stored in liquid nitrogen for many years frozen in TC-100 or similar medium supplemented with heat inactivated 10 % FBS. When needed, frozen cells may be thawed in a carefully designed protocol to avoid damage.
6. Cleanliness of glassware is an important requirement. Insect cells are very sensitive to contaminants, especially detergents. Flasks should be washed using ~1 % Triton X-100.

Insect cell cultures are widely applied in a variety of scientific fields. They are also used for the production of recombinant proteins, viral pesticides, and vaccines, as well as for basic research on morphogenesis, genetics, virology, pathology, biochemistry, endocrinology, and molecular biology. Cell lines have been established from several orders of insect including Diptera, Lepidoptera, and Hemiptera. Dipteran and lepidopteran cell lines are particularly important in agriculture and biotechnology and will be discussed in more detail below.

The first breakthrough in cell line development from arthropods was spindle-, round-, and crescent-shaped cells from the ovarian tissues of the Gum Emperor moth, *Antheraea eucalypti* Scott (available as ATCC® CCL-80™), a native of Australia and New Zealand [51]. These were grown on Grace's insect tissue culture medium, which is a modified Wyatt medium [52] supplemented with ten vitamins. This medium gained acceptance in research and commercial applications, not just because it supports the growth of cells in vitro but also because of its usefulness in the establishment and maintenance of many insect cell lines [53, 54]. Since the introduction of Grace's medium, there has been a geometric upsurge in the study of ICs leading to the establishment of over 500 cell lines from different insect species and orders [55–57].

More recently, interest has been focused on developing new lepidopteran cell lines for potential application in biotechnology, largely thanks to advances made in recombinant DNA technology starting in the early 1970s which has led to the production of new recombinant proteins and genes useful in public health and agriculture. Lepidopteran cell lines have been established primarily to propagate insect viruses as biopesticides for the biological control of insect pests [58]. Lately, the baculovirus expression vector system combined with insect cell cultures (IC-BEVS) has become more attractive for the expression of many heterologous proteins than those derived from bacteria, yeast, vertebrates, or viruses due to its unique characteristics. This technology is also being used for the construction of recombinant BVs to use as biopesticides, which offer comparatively faster killing of insect pests than wild-type BVs [59–62].

Insect cell lines have become invaluable in:

(a) The study of virus–cell interactions [63–66]. This has enhanced the application of insect cell lines as a tool in basic research.
(b) Virus-related research for the development of viral pesticides [67, 68]. This is the cornerstone of the application of insect cell lines in agriculture.
(c) The production of recombinant proteins/vaccines [69]. This constitutes the foundation for application of insect cell lines in biotechnology.
(d) The development of health-related products using vectors developed from insect viruses, especially BVs (with the major genus being the nucleopolyhedrosis viruses [NPVs]) [70]. This has defined the application of insect cell lines for the control of vectors of human diseases and has had great impact in health and disease.

## 8.5 Insect Cell Line Development, Maintenance, and Production

Given the importance of insect cell culture as a foundation for modern insect biotechnology and, importantly, the production of recombinant proteins, the following section will discuss briefly the media and methods of insect culture development, maintenance, and production.

Generally, nutrient requirements of ICs and those of vertebrate cells are essentially similar, although there are a few differences that must be considered when designing insect cell culture media. The following conditions are present in insect hemolymph and must be met for the formulation of successful culture media:

(a) A high level of amino acids [49, 71]
(b) High levels of free organic acids such as citrate, succinate, oxalate, or malate, which range from 0.1 to 30 mmoles per insect [49, 72]
(c) Acidic conditions in a normal range of pH 6.2–pH 6.9, higher than typically found in mammalian tissue fluids [49]
(d) Supplementation with metabolic sources of cell membrane components and the steroid hormone, ecdysone, because ICs lack the capacity to make steroids [49, 73]
(e) Osmolarity in the range of 340–390 mOsmol/kg, more than twice as high as that of the vertebrate blood [74]
(f) High concentrations of metabolites in the glutamine and glucose metabolism pathways [72, 73]

As indicated previously, Grace's medium has always been one of the most popular media for insect cell culture. The formulation was designed to mimic the chemical composition of the hemolymph from *B. mori*. Prior to use, the medium is typically supplemented with fetal calf serum, yeast extract, lactalbumin hydrolysate, and bovine serum albumin in varying combinations and amounts. Since Grace's medium, other media have been developed for specific uses in insect biotechnology. For example, ESF 921 medium was developed to address the need for a single medium that can support high cell growth using Sf9, Sf21, *Trichoplusia ni* (e.g., High Five™ and Tn 368 cells), and *Drosophila* cells (e.g., S2). High levels of recombinant protein have been expressed in ESF 921 using a variety of cell lines with insect cell baculovirus expression vector system (IC-BEVS; see below) technology or the stable transformed cell technology. ESF 921 was designed to especially enhance the expression of glycosylated proteins. Sf9 and Sf21 were developed from *Spodoptera frugiperda* ovarian tissue, while BTI-Tn5B1-4 (High Five™) and S2 were developed from *T. ni* embryonic tissue and *Drosophila*, respectively.

Insect cell lines are typically cultivated in steps depending on the magnitude of ultimate deployment (see Sect. 7.7). The steps include:

(a) Shaking in flasks containing serum-free medium to loosen adherent cells
(b) Growth in 2.5–30 L perfusion bioreactor or up to 200 L (where large scale is needed)
(c) Primary separation as part of downstream processing
(d) In-process analytics
(e) Large-scale protein purification for protein expression experiments

The maintenance of high-quality ICs is absolutely important for the success of the all-important IC-BEVS. Comparatively, mammalian cell lines do not deteriorate as fast as insect cell lines, which calls for strict maintenance. As already indicated, most researchers routinely use *S. frugiperda* Sf9 and Sf21 cell lines (Sf9 is a subclone of Sf21). It is advisable to start cells from a frozen cell stock which needs to be pre-warmed to room temperature. This is to avoid contamination, over-passage

of cells, and negligence in maintenance resulting in slower division with time. It is recommended that pre-warmed cultures be diluted about 1:10 to a density of $1 \times 10^6$/ml and grown on TC-100 medium supplemented with 10 % FBS [73–75].

## 8.6 Development and Application of the Insect Cell Baculovirus Expression Vector System (IC-BEVS)

The commonest type of insect viruses is the BVs. BVs are known to infect over 600 insect species worldwide. Members of 11 families are pathogenic to insects. BVs are usually associated with the insect arthropod orders of Lepidoptera, Hymenoptera, Diptera, Neuroptera, Coleoptera, Trichoptera, Crustacea, and Acarina (mites). The virions are rod shaped, 40–70 nm × 250–400 nm, comprising a lipoprotein envelope surrounding a protein capsid containing a DNA–protein core. NPVs include those that infect the lepidopterans, *Helicoverpa armigera*, *S. litura*, *S. exigua*, *Amsacta moorei*, *Agrotis ipsilon*, *A. segetum*, *Anadividia peponis*, *T. ni*, *Thysanoplusia orichalcea*, *Adisura atkinsoni*, *Plutella xylostella*, *Corcyra cephalonica*, *Mythimna separata*, and *Phthorimaea operculella* [76]. Those that infect caterpillars of lepidopteran insects and dipteran larvae appear to have attracted the greatest attention. Each BV is highly specific in its host range, being limited to a single type of insect. The unique characteristics of BVs have enabled the development of the IC-BEVS, which has found great use in biotechnology. IC-BEVS are safe and lend themselves to abundant and rapid production of recombinant proteins in ICs. The development of the first(?) IC-BEVS is credited to MD Summers, who was granted a patent for it in 1988. The IC-BEVS has been widely used in research and scientific industrial communities for the production of high levels (up to 1000 mg/mL) of correctly posttranslationally modified (folding, disulfide bond formation, oligomerization, glycosylation, acylation, proteolytic cleavage), biologically active, and functional recombinant proteins [77]. The IC-BEVS is based on the introduction of a foreign gene into a region of the genome which is nonessential for viral replication via homologous recombination with a transfer vector containing the target gene. The resulting recombinant BV lacks one of several nonessential genes (*polh*, *v-cath*, *chiA*, etc.) which is replaced with a foreign gene encoding a heterologous protein which can be expressed in cultured ICs and insect larvae.

Because of their capacity to produce many recombinant proteins at high levels and also provide significant eukaryotic protein processing capabilities, IC-BEVSs have extended the frontiers of basic research. Furthermore, important technological advances over the past 20 years have improved upon the original methods developed for the isolation of BV expression vectors. Today, virtually any investigator with basic molecular biology training can isolate a recombinant BV vector relatively quickly and efficiently and use it to produce a desired protein in an insect cell culture [78]. The IC-BEVS has become a core technology for:

- The cloning and expression of genes for the study of protein structure, processing, and function
- The production of biochemical reagents

- The study of regulation of gene expression
- The commercial exploration, development, and production of vaccines, therapeutics, and diagnostics
- Drug discovery research
- The exploration and development of safer, more selective, and environmentally compatible biopesticides consistent with sustainable agriculture

The most widely used lepidopteran cells for IC-BEVS are Sf9 and Sf21 cell lines and the High Five™ cell line, designated BTI-Tn5B1-4. Sf9 cells are a subclone of the Sf21 cells and were selected for their faster growth rate and higher cell densities than the Sf21 cells; both are preferred for virus expansion. Sf21 cells can compare favorably, in terms of heterologous protein expression, to both High Five™ and Sf9 cell lines in certain situations. Figure 8.2 presents a general scheme for BEVS development [73, 79, 80].

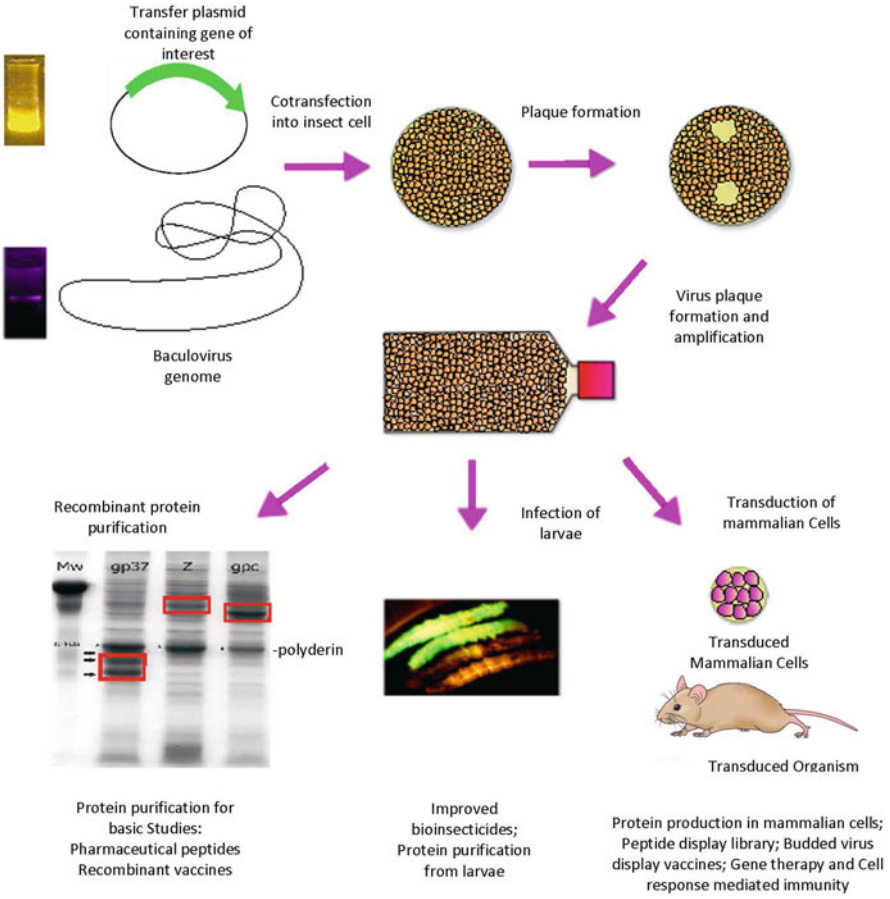


**Fig. 8.2** General process for baculovirus cloning. Genomic DNA and a transfer plasmid are cotransfected into an insect cell culture. Recombinant virus propagates [73]

## 8.7 Applications of IC-BEVS in Industrial Processes

The BV vector most commonly used in industry and research laboratories for recombinant protein production is based on the *Autographa californica* multinuclear polyhedrosis virus (AcMNPV) with Sf9, Sf21, or High Five™ cells; whole *T. ni* insect larvae are also used as suitable expression hosts [79–81].

In recent times, IC-BEVS has also gained prominence and wide acceptance for routine production of recombinant proteins in ICs and larvae [82–86]. Furthermore, IC-BEVS is applied in the development of strategies for displaying foreign peptides and proteins on virus particles and the insertion of mammalian cell-active expression cassettes in BVs to express genes efficiently into many different mammalian cell types. BVs engineered to display foreign peptides and proteins on the viral surface have proven particularly useful as immunogens [87]. The Sf9 and BTI-Tn5B1-4 cell lines described previously, as well as those trademarked by Invitrogen as High Five™ cells, are among the flagship cell lines for producing viruslike particle (VLP) vaccines with recombinant IC-BEVS. VLPs are multi-protein structures that mimic the organization and conformation of authentic native viruses but lack the viral genome, potentially yielding safer and cheaper vaccine candidates [88]. Insect biotechnology, through the development of IC-BEVS, has really revolutionized health and biotechnology industry, as shown in Fig. 8.3 and described in subsequent sections.

### *8.7.1 Vaccines and Vaccination*

Two animal vaccines are presently on the market, and several immunotherapeutic and human vaccines are being developed and produced in Sf21, Sf9, expreSF+™, or High Five™ cells. In Europe, two commercial subunit vaccines for classical swine fever are produced in *S. frugiperda* cells by Intervet, Leiden, The Netherlands [89]. Several human vaccines are being produced in *S. frugiperda.* They include:

(a) Provenge® (sipuleucel-T), an immunotherapeutic vaccine for prostate cancer developed by Dendreon Inc, Seattle, WA
(b) Flublok®, a vaccine for human influenza virus developed by Protein Sciences Inc., Meriden, CT
(c) Chimigen® vaccines for chronic hepatitis B and C developed by ViRexx Medical Corp., Calgary, Canada

Commercial products developed using *T. ni* High Five© cells include:

(a) FavId® (idiotype/KLH), an immunotherapeutic vaccine for B-cell non-Hodgkin's lymphoma developed by Favrille Inc., San Diego, CA.
(b) Cervarix®, a vaccine for cervical cancer developed jointly by MedImmune, Gaithersburg, MD, and GlaxoSmithKline, Rixensart, Belgium. Cervarix is the first vaccine produced in ICs to be commercialized for human use.

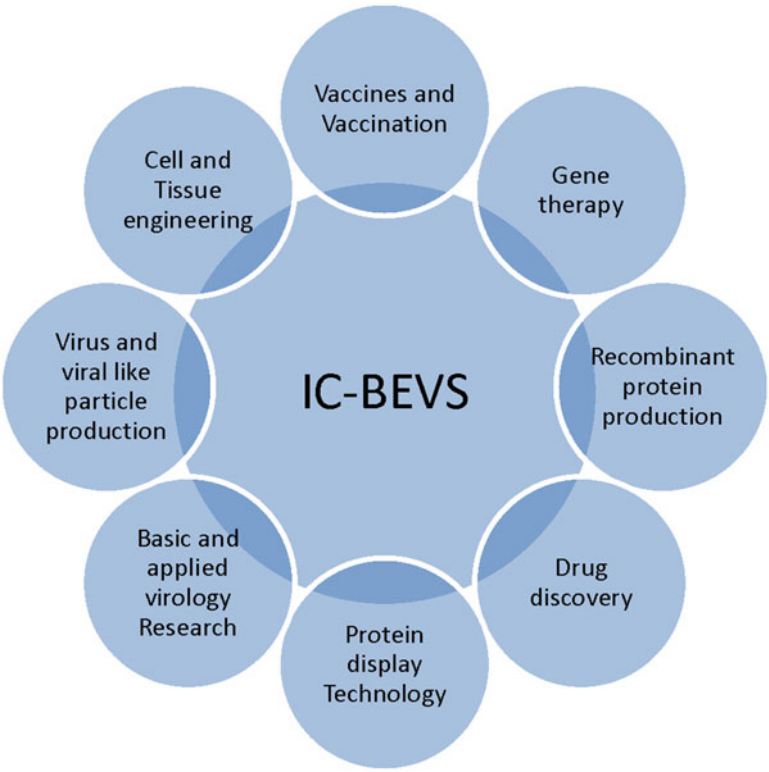


**Fig. 8.3** Applications of insect cell baculovirus expression vector systems (IC-BEVS)

The commercial availability and probable success of vaccines for animal or human use in the near future will provide even greater impetus for the application of the BV-insect cell technology in research and medicine.

### *8.7.2 Gene Therapy*

The pioneering studies of Hofmann [90] and Boyce [91] and their coworkers in the mid-1990s, in which they outfitted AcMNPV with an expression cassette driving a marker gene via a strong viral promoter (active in the target cells), boosted interest in BVs as potential vectors for gene therapy. A well-known and accepted economic process for this purpose is the production of recombinant adeno-associated virus (rAAV) vectors in lepidopteran Sf9 cells using recombinant AcMNPV or other BVs [92–96]. BV-mediated gene transfer has already demonstrated therapeutic efficacy in ex vivo and in vivo gene therapy studies [97–100]. Gene therapy has so fully come of age so much that the high potential of this therapeutic modality can now be

realized for treating several diseases, such as severe immune deficiencies [101, 102], ocular diseases [103], and cancer [104]. Several virus-derived vectors and non-virus gene transfer agents have been used to address these applications. Lentiviruses (LVs) have become widely used for gene delivery and gene therapy when sustained gene expression is required [105]. The application of BV for producing AAV and LV facilitates the development of the gene therapy field from the bioprocess perspective. Currently, BV transduction has been used to generate stem cells carrying therapeutic payloads of interest suitable for cartilage and bone tissue engineering and targeted glioma gene therapy [106]. Effective transduction and high-level transgene expression mediated by baculoviral vectors are especially suited for cancer gene therapy [107]. Baculoviral vectors armed with suicide genes, tumor suppressor genes, proapoptotic genes, immunopotentiating genes, and anti-angiogenesis genes have been tested in vivo in animal tumor models in many different anticancer regimens [108–110]. More recently, ex vivo gene therapy studies have been conducted using BVs equipped with suicide genes to transduce stem cells [112–114]. This approach benefits from the tropism exhibited by many adult stem cells for primary, solid, and metastatic tumors. Kotin and coworkers have extended the versatility of the IC-BEVS to include the production of faithfully assembled and packaged rAAV vectors suitable for in vivo gene therapy applications [115].

### *8.7.3 Recombinant Protein Production*

As already stated, the IC-BEVS is one of the most powerful, robust, and versatile eukaryotic expression systems available [116]. Given its speed of development and versatility for the expression of a wide range of protein families, the IC-BEVS offers multiple advantages for protein production in a variety of applications. Since the development of the IC-BEVS in the 1980s [117], thousands of recombinant proteins, ranging from cytosolic enzymes to membrane-bound proteins, have been successfully produced in BV-infected ICs. However, expression yields in optimized transformed fed-batch mammalian cells cultured in a bioreactor commonly reach grams of recombinant protein per L; in ICs infected by recombinant BVs, the yield rarely exceeds 50–100 mg per L. This relatively low expression capacity can be compensated in the IC-BEVS by the short development times and lower costs associated with a specific product. This implies that for the production of a recombinant protein with market needs not exceeding 5–10 kg per year (i.e., subunit vaccines), the IC-BEVS is currently one of the best alternatives [118]. In fact, most licensed products obtained in ICs correspond to vaccines and not to products with a high production demand such as therapeutic antibodies [119]. However, the yield is not the only bottleneck of the IC-BEVS. A marked proteolysis of recombinant proteins during BV-based production is frequently encountered. This observation is due, in part, to the cytopathogenic effects of the BV vectors in ICs during infection [69, 93, 94].

The in-depth understanding of both uninfected and infected insect cell physiology using metabolic engineering and the constant improvement of insect cell media and culture techniques are bound to contribute to the rational design and validation of robust, scalable, and safe production processes leading to improved yields based on the IC-BEVS technology.

### *8.7.4 Drug Discovery*

Information abounds in the scientific and biotechnology literature about significant advances in BEVS technology which has led to the production of more than 1000 viral, prokaryotic and eukaryotic recombinant proteins. Many biotechnology initiatives and start-up industries have arisen in the USA and the rest of the industrialized world based on the BEVS and its applications for discovery, development, and commercial production areas for medicine and agriculture [85]. To achieve the desired success, drug and vaccine discovery must take into account that large amounts of complex eukaryotic proteins are produced with appropriate posttranslational processing and have desired biological activity. Fortunately, BEVS provide the platform to achieve this cheaply and fast. The phenomenal applicability of BEVS technology in drug discovery has been accentuated by the ability to crystallize medically important proteins, to advance the understanding of three-dimensional structure, and to enhance the precise design of drugs that are specifically targeted for specific intervention in many diseases.

Examples of the application, efficiency, and cost-effective BEVS technology in the rapid development of drugs include an experimental vaccine for the deadly Hong Kong "bird flu" virus (H5N1). The first of this vaccine, Pandemrix, was approved in 2013 by the US Food and Drug Administration and rapidly manufactured by GlaxoSmithKline PLC (GSK) [95]. Currently, no definitive medication protocol specific to severe acute respiratory syndrome (SARS) has been developed; however, there has been a rapid development of a test SARS vaccine upon request by the US National Institutes of Health. Protein Sciences Corporation (PSC) used BEVS technology to develop and deliver 1700 doses of an experimental "bird flu" vaccine in 2 months. PSC has also developed Flublok®, the world's first recombinant protein-based seasonal influenza vaccine based on the BEVS technology [96].

In 2013, 30 years after its inception, the baculovirus–insect cell expression system and its associated technologies were celebrated. This system still forms a mainstream platform for the production of recombinant proteins for fundamental and applied science. Scientists are encouraged to take this system further into the next decades by overcoming remaining challenges, e.g., to optimize BEV genome composition, to improve genome stability in order to guarantee product quality over batches, and to simplify downstream processing without losing safety. This will allow the baculovirus expression system to become the system of choice for many applications based on its convenience and the yield, quality, and safety of the recombinant products [97].

### *8.7.5 Protein Display*

In addition to the numerous advantages and usefulness of IC-BEVS already discussed, they have become one of the systems of choice to display vectors that would be suitable for targeting and gene transfer to mammalian cells. Display of heterologous proteins is usually carried out using mammalian viruses such as retro-, adeno-, and adeno-associated viruses [98, 85]. The main advantage of molecular display technology is the physical linkage between the proteins or peptides displayed (phenotype) and the genes that encode them (genotype). This allows simultaneous selection of the genes with proteins of the desired affinity or function [99]. Among extremely versatile display technologies are those based on the IC-BEVS. The expression of foreign proteins on the BV surface was described previously. The applicability of IC-BEVS display technology in the generation and screening of eukaryotic expression libraries has been eminently demonstrated and established [100, 101]. The first proteins chosen to be displayed were glutathione S-transferase and the external domain of gp120, a surface glycoprotein of human immunodeficiency virus type I (HIV-1). Both were successfully expressed on the BV 25 surface as N-terminal fusions to the mature gp64 protein; additionally, gp120 was shown to be functional in binding to its ligand, CD4 [102]. Variations of the HIV-1 gp41 epitope, "ELDKWA," were BV-infected ICs and screened with a specific neutralizing monoclonal antibody. In 1997, Grabherr and colleagues demonstrated the presentation of the ectodomain of the HIV-1 gp41 envelope protein on the virus surface, either as a fusion to the entire gp64 or, alternatively, to its transmembrane domain [101, 100]. Other BEVS-based systems include:

1. Immunization of mice with recombinant baculovirus (rBV) displaying gp64 amino-terminal fusion proteins for the nuclear receptors, liver X receptors (LXRs) and farnesoid X-activated receptor (FXR). This is used to develop monoclonal antibodies to LXR and FXR. LXRs are ligand-activated transcription factors of the nuclear receptor superfamily. LXR activation normalizes glycemia and improves insulin sensitivity in rodent models of type 2 diabetes and insulin resistance. FXR is a member of the nuclear receptor superfamily. It has emerged as a key player in the control of multiple metabolic pathways. On its activation by bile acids, FXR regulates bile acid synthesis, conjugation, and transport, as well as various aspects of lipid and glucose metabolism.
2. Display of the green fluorescent protein (GFP) and envelope glycoproteins E1 and E2 of rubella virus on the BV surface [103, 104].
3. The use of BV surface display for the presentation of antigenic sites from the foot-and-mouth disease virus [104] and the p67 antigen of *Theileria parva*, the causative agent of the cattle disease East Coast fever [105].
4. The use of a display strategy in which enhanced green fluorescent protein (EGFP) was fused with either the N- or C-terminus of AcMNPV major capsid protein vp39, leading to the high-level incorporation of the EGFP–vp39 fusion into the BV capsid with apparent compatibility with oligomeric proteins [106, 107].

### 8.7.6 IC-BEVS in Basic Virology Research

The field insect biotechnology based on cell lines and IC-BEVS has grown to the extent that now over 500 insect cell lines have been established from many insect species representing numerous insect orders and from several different tissue sources. These systems are used as research tools in virology, to study signaling mechanisms in insect immunity and hemocyte migration and to test the hypotheses about gene expression, and in screening programs designed to discover the mode of action of new insecticides. Such virology research is revealing fundamentally new information on virus/host cell interactions and uncovering signal transduction pathways that are new to insect science. Research based on IC-BEVS is leading to the development of high-speed screening technologies which are essential in the search for new insect pest management tools. In addition to the use of a few insect cell lines designed to produce proteins of biomedical significance in routine industrial processes, as described previously, both primary cell cultures and established lines are used in basic biological studies to reveal how ICs work.

The continued development of new cell culture technology is essential for the continuously growing application of BV biotechnology. Cell lines used for academic research are currently dominated by the same ones used for commercial purposes described earlier, namely, the *S. frugiperda* line, Sf21, and its clonal isolate, Sf9, and the *T. ni* line, BTI-Tn5B1-4 or High Five™ cells, thus, the long-held prediction that the immense potential application of the IC-BVS as a tool in cell and molecular biology, agriculture, and animal health has been achieved [108, 85]. The versatility and recent applications of this popular expression system have been demonstrated by both academia and industry, and it is clear that this cell-based system has been widely accepted for biotechnological applications. Numerous small to midsize start-up biotechnology companies in North America and Europe are currently using IC-BEVS technology to produce custom recombinant proteins for research and commercial applications. The recent breakthroughs in the development of several commercial products that will impact animal and human health, as noted above, will further enhance interest in this technology for pharmaceutical applications [108, 85].

### 8.7.7 Virus and Viruslike Particle Production

As noted previously, the ability to make a large variety of VLPs has been successfully achieved in the IC-BEVS/insect cell system [109]. The production and scale-up of these particles are currently being addressed, mostly for candidate vaccines and as delivery agents for use as therapeutics. Recently, as noted, AAV vectors, which can be potentially used for human gene therapy, have been produced in ICs using three BV vectors to supply the required genes. The use of the host IC allows mass production of VLPs in a proven scalable system. This chapter focuses on the

methodology, based on the work done by various laboratories, for the production of AAV-like particles and vectors in a BEVS/insect cell system [110]. New BV–AAV reagents have made it possible to produce rAAV using two or three different BVs. Using this multiplicity of infection (MOI), strategy improves the chances of producing a reengineered two-BV system in benchtop-scale production. The percentages of rAAV producer cells increase at higher MOIs. Production of the reengineered cells can also be done at pilot/large scales with MOIs of ≤1. The downstream processing methods (not discussed in this section) depend on the scale of production.

#### 8.7.7.1 Benchtop-Scale Production

Sf9 cells are grown in benchtop shaker incubators at 27–28 °C with agitation to provide sufficient dissolved $O_2$ in the medium. This is needed to maintain the cells in exponential growth phase which is ideal for rAAV production. Small culture-volume rAAV preparations are routinely performed by either triple infection (Rep baculovirus, Cap baculovirus, and vector genome baculovirus) or double infection (Rep-Cap baculovirus plus the vector genome baculovirus). AAV vector may be harvested directly from the shake flask when cell viability decreases to <30 % by freeze–thaw lysis. The cell lysate may then be nuclease-treated to reduce viscosity and digest extravirion DNA. Debris is removed by centrifugation or filtration and the rAAV is concentrated by adding polyethylene glycol (PEG, 8000 MW) to the clarified lysate (2 %, w/v) and enhancing the precipitation of the rAAV particles [70, 109, 110].

#### 8.7.7.2 Pilot- or Large-Scale Production

Production of IC-BEVS at pilot or large scale is done in stirred tank bioreactors and can only be limited by the capacity of the available upstream and downstream processing systems. Commercially available media include serum-free formulations from several manufacturers. As in the benchtop scale, consistently regulated temperature, agitation, and dissolved oxygen are the most critical parameters for promoting cell growth and rAAV productivity. The buffering capacity of these commercial growth media adequately maintains the acidity of the culture within levels acceptable for the growth of Sf9 cells, making pH monitoring optional. The low-MOI strategy used for heterologous protein expression in the baculovirus–insect cell system has proved very useful for rAAV production [111, 112]. Negrete and colleagues [76, 77] found that using MOIs much less than 1 was effective for rAAV production if the cell density at the time of infection was appropriately adjusted to accommodate the increased time until baculovirus-induced cell cycle arrest. Initially, few cells are infected, and the probability that different viruses coinfect a cell is near zero. Thus, using a primary MOI of 0.05, the MOI of the secondary round is about 5 [113, 114].

### 8.7.8 *Use of Baculoviruses in Cell Therapy and Tissue Engineering*

Tissue engineering is the use of a combination of cells, engineering, and material methods, together with suitable biochemical and physicochemical factors, to improve or replace biological functions. While it was once categorized as a subfield of biomaterials science, having grown in scope and importance, it can be considered as a field in its own right. The term is closely associated with applications for repairing or replacing portions or whole tissues (i.e., the bone, cartilage, blood vessels, bladder, skin, muscle, etc.). Great success has been achieved in the area of bone marrow mesenchymal stem cells which are considered a highly potent vehicle for cell-based therapies [115] due to their differentiation potential and special immunological properties such as suppression of the host graft response. BV transduction of mammalian cells does not seem to seriously compromise their immunological status and behavior in vitro and, more importantly, in vivo [116, 117]. BVs are potentially safe candidates for therapeutic gene delivery because of their intrinsic inability to replicate in mammalian cells and their low cytotoxicity [53, 68, 118–120]. Articular cartilage is a heavy tissue that protects bones, but is limited in self-repair capacity. Hu and coworkers first demonstrated that BV can effectively transduce rat articular chondrocytes [86, 121–123]. However, chondrocytes tend to dedifferentiate during subculture, thus hindering their in vitro expansion and subsequent transplantation back to the host. Sung and colleagues demonstrated that transduction of dedifferentiated chondrocytes with a BV vector (Bac-CB) expressing bone morphogenetic protein 2 (BMP-2) not only restores the differentiation status of passaged chondrocytes in vitro but also increases accumulation of cartilage-specific extracellular matrix [124]. These activities were further augmented by BV-mediated coexpression of transforming growth factor β [125, 126]. Lin and colleagues recently constructed a recombinant BV (Bac-CV) expressing vascular endothelial growth factor (VEGF) [82, 127, 128]. Implantation of Bac-CV- and Bac-CB-transduced rabbit bone marrow-derived mesenchymal stem cells into critical-size segmental bone defects at the femora of New Zealand White (NZW) rabbits accelerated and ameliorated bone healing, thanks to the in vivo coexpression of *BMP-2/VEGF*, ensuing improved osteogenesis and angiogenesis [129–131].

## 8.8 Conclusion

The production of bioinsecticides and recombinant proteins for use in basic research, diagnostics, and biomanufacturing are well established and are among the numerous benefits of insect biotechnology to mankind, especially propelled by IC-BEVS. As a result of new applications of the technology in human and animal health, including biopharmaceuticals and new-generation vaccines, the IC-BEVS platform has become the technology of choice for virologists worldwide. The technology is also

seamlessly suited for producing viral vectors for cell therapy and for readily expressing proteins of interest in fundamental biological research, tissue engineering, drug discovery, sustainable agriculture, and other applications. The continual improvement of tissue culture techniques that promote high-quality cell systems as well as the use of BV as a display system is building on the improvement of the quality of insect cell media and efficiency of cultivation. Generally, insect biotechnology is a most auspicious development in modern scientific and biotechnological pursuits leading to scalable and safe IC-BEVS-dependent biomanufacturing processes.

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